December 2016

Exendin 4 Conjugation and Sequence Modification to Treat Type 2 Diabetes and Obesity

Ronald Bonaccorso Bonaccorso
Syracuse University

Abstract

This thesis addresses three primary questions based on the pharmacodynamics (PD) or pharmacokinetics (PK) of the diabetes drug exendin 4 (Ex-4), and vitamin B_{12} (B_{12}) bioconjugates, thereof.

Q1. (Chapter 2) What effect does B_{12} conjugation to Ex-4 have on agonism of the glucagon-like peptide-1 receptor (GLP-1R) in vitro and on PD/PK (including brain uptake and function) in vivo?

Goal: To remove side-effects (nausea, weight loss) of Ex-4 without loss of glucoregulation.

Q2. (Chapter 3) Can B_{12} dietary uptake proteins such as gastric intrinsic factor offer protection to B_{12} conjugated peptides or proteins (focusing on Ex-4), with a view to improving in vivo PK?

Goal: To demonstrate (in vivo) that IF binding of B_{12}-Ex-4 confers protection against gastric proteolysis as a road-map to oral peptide delivery.

Q3. (Chapter 4) Can a dual agonist of the GLP-1R and neuropeptide Y2 receptor, based on the Ex-4 primary amino acid sequence, be designed and validated in vitro and in vivo.

Goal: To create a new therapeutic to simultaneously treat diabetes and obesity.

Exendin 4 Conjugation and Sequence Modification to Treat Type 2 Diabetes and Obesity

By

Ronald L. Bonaccorso

B.S. Chemistry, Rochester Institute of Technology 2012

DISSERTATION

Submitted in partial fulfillment for the degree of

Doctor of Philosophy in Chemistry

Syracuse University

December 2016

Contents

Chapter 1

Abbreviations

1	Cyanocobalamin
2	B_{12}-CA; B_{12}-carboxylic acid
3	B_{12}-propargyl amine
4	B_{12}-amino butyne
5	B_{12}-amino pentyne
6	B_{12}-amino hexyne
7	B_{12}(3)-Ex-4
8	B_{12}(4)-Ex-4; B_{12}-Ex-4
9	B_{12}(5)-Ex-4
10	B_{12}(6)-Ex-4
11	RLB001; HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQRY-NH2
12	RLB002; HGEGTFTSDLSKQMEEEAVRLFIEWLRHYLNLVTRQRY-NH2
13	RLB003; IKPEAPREDASPEEENQAYKEFIAYLNLVTRQRY-NH2
14	RLB004; HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSRHYLNLVTRQRY-NH2
15	RLB005; HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQ-NH2
16	RLB006; HGEGTFTSDLSK(azido)QMEEEAVRLFIEWLKNGGPSSTRQRY-NH2
B_{12}	Vitamin B_{12}
GIT	Gastrointestinal tract
IF	Intrinsic factor
DMB	5,6-dimethylbenzimididazol

COA	Coenzyme A
THF	Tetrahydrofolate
HC	Haptocorrin
MRP	Multidrug resistant protein 1
TCII	Transcobalamin II
CD320	Cluster of differentiation 320
Cubam	Cubilin-amnionless
BBB	Blood brain barrier
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
GSIS	Glucose stimulated insulin secretion
BMI	Body mass index
DPP-IV	Dipeptidyl peptidase IV
GLP-1	Glucagon-like peptide-1
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1R	GLP-1 receptor
GLP-2R	GLP-2 receptor
GIPR	GIP receptor
GCGR	Glucagon receptor
Ex-4	Exendin 4
PD	Pharmacodynamics

PK	Pharmacokinetics
EC_{50}	Effective concentration 50
GLP-2	Glucagon-like peptide-2
GPCR	G protein-coupled receptor
cAMP	Cyclic adenosine monophosphate
PYY	Peptide Tyrosine Tyrosine
NPY1R	Neuropeptide Y1 receptor
NPY2R	Neuropeptide Y2 receptor
IBX	2-iodoxybenzoic acid
HYP	2-hydroxypyridine
MALDI-ToF-MS	Matrix-assisted laser desorption-ionization time of flight mass spectrometry
EDC	1-ethyl-3-(3-3-dimethylaminopropyl)carbodiimide
HOBt	Hydroxybenzotriazole
DMSO	Dimethyl sulfoxide
DMF	Dimethyl formamide
RIP1	Rat insulin 1 promoter
CRE	cAMP response element
Luc	Luciferase
AKAR3	A kinase activity reporter
FRET	Fluorescence resonance energy transfer
A.C.	Adenylate cyclase

HEK-293	Human embryonic kidney cells
PKA	Protein kinase A
YFP	Yellow fluorescent protein
CFP	Cyan fluorescent protein
FHA	Forkhead associated
ATP	Adenosine triphosphate
CHO	Chinese hamster ovary
BW	Body weight
AN	Arcuate nucleus
ESMS	Electrospray mass spectrometry

Chapter 1: Introduction

1.1 Vitamin B_{12} (B_{12})

Vitamin B_{12} (B_{12}) is one of thirteen essential vitamins and the most recently discovered (see Figure 1).[1] B_{12} is a deep red color and has been referred to as "nature's most beautiful cofactor".[2] B_{12} is in a rare category as a naturally occurring organometallic and was the first such compound discovered.[3,4] Humans cannot produce B_{12}, as it is made only by certain prokaryotes such as *Pseudomonas denitrificans,* and so we must acquire it entirely through diet.[5,6,7,8,9,10] B_{12} is a water soluble molecule and readily degraded in the presence of acid.[11] Humans employ a complex system of binding proteins and receptors in order to navigate the human gastrointestinal tract (GIT) and deliver B_{12} into the blood stream, then proliferating cells, including those in the brain. A typical human takes in 1 -5 µg per day of B_{12} but the body can store B_{12} in the liver for up to five years and a deficiency can take years to discover.[12] Deficiency is typically the result of a defect relating to the production of binding proteins such as intrinsic factor (IF). There are no known cases of overdosing from B_{12} and no recommended upper limit in dosage.[13]

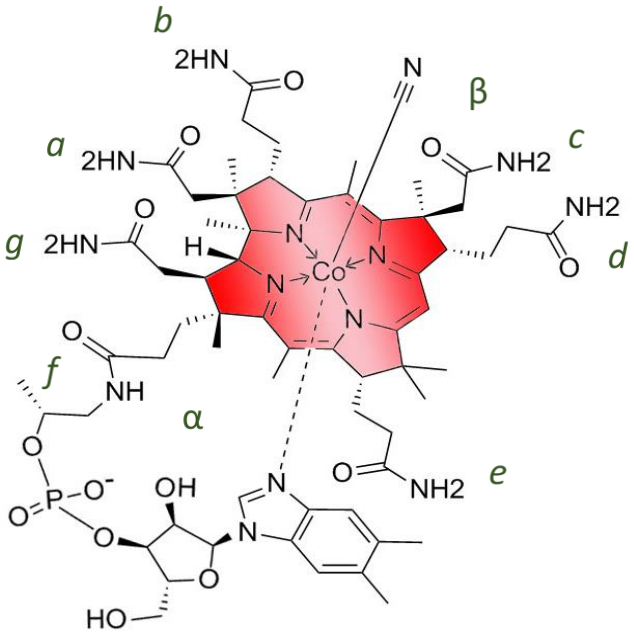


Figure 1. B_{12} (cobalamin) structure, shown as cyanocobalamin.

The core of B_{12}, also known as cobalamin, is comprised of a corrin ring chelating a cobalt(III) ion. This corrin ring, which is responsible for the red color of B_{12}, has two sides, α and β (see Figure 1). The corrin ring is similar to a porphyrin ring with key differences being the lower saturation in the corrin (3/4 vs 4/4) and the greater asymmetry of the corrin due to the loss of a methylene bridge between pyrrole rings C and D (also resulting in one less carbon (19) compared to the porphyrin (20)).[14] On the α- side there is a 5,6-dimethylbenzimidazole (DMB) coordinated to the cobalt ion through a nitrogen atom. Off this DMB is a ribose moiety connected to the corrin ring through a phosphate and amide linker. Although there are several functional groups located on B_{12}, conjugations, made at the 5'-hydroxyl of the ribose moiety, maintain the most significant binding affinity by B_{12} binding proteins.[15,16]

1.1.1 History of B_{12}: Discovery and Diseases

Symptoms of pernicious anemia, a disease characterized by pallor, sharp pains and excessive urination, were first described by physicians in the 1800s.[17,18] Significant progress toward a cure for the disease, which was typically fatal at the time, did not come until the 1900s. The first major breakthrough was published in 1925 when Whipple, through research on anemia induced by blood loss in dogs, discovered that a diet composed of liver, particularly raw bovine liver, helped the dogs recover.[19,20] This led to the first idea of B_{12}, which was that there had to be something present in liver that was promoting recovery in pernicious anemia. Minot and Murphy then showed in 1926 that 45 patients with pernicious anemia given a daily meal consisting of bovine liver for several months, significantly improved.[21] The discovery of effective pernicious anemia treatment won Minot, Murphy and Whipple the Nobel prize in chemistry in 1934.[22]

Castle set out to address why liver was effective in treating pernicious anemia. His discoveries, through a set of experiments published in 1929 and 1930 using gastric juices from patients with and

without pernicious anemia, led him to believe that the meat had some factor that could only be utilized if those juices were present.[23,24] What Castle realized was that, although the treatment to pernicious anemia was extrinsic factor (B_{12}) and the cause was B_{12} deficiency, the real problem was a lack of IF, which rendered the body unable to access B_{12}.

The compound responsible for the Nobel prize in 1934 wasn't isolated until over a decade later.[25] In 1947, B_{12} was simultaneously crystallized by Folkers at Merck and Smith at Glaxo and published in Nature and Science, respectively.[26,27] The second Nobel prize for B_{12} was awarded in 1964 to Dorothy Hodgkin for determining the single-crystal X-ray structure of B_{12} in 1955.[28]

1.1.2 Metabolism of B_{12}

B_{12} acts as a cofactor in two enzymatic processes (see Figure 2). The first, with cytosolic methionine synthase, is the conversion of homocysteine to methionine, and the second, with mitochondrial methylmalonyl coenzyme A (COA) mutase, is the conversion of methylmalonyl-COA to succinyl-COA.

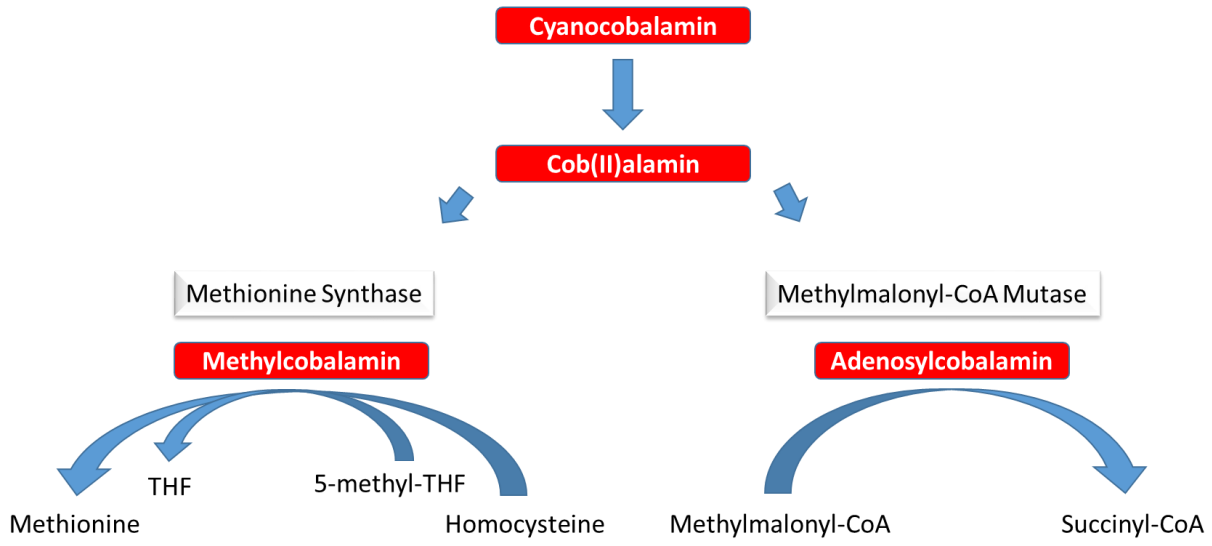


Figure 2. Representation of B_{12} coenzyme function in methionine synthase and methylmalonyl-COA synthase.

As the coenzyme for methionine synthase, a methyl group is transferred to homocysteine from 5-methyl-tetrahydrofolate (5-methyl-THF) producing methionine and THF. During this transfer B_{12} plays a role in remethylating homocysteine and accepting a methyl group from 5-methyl-THF. In the absence of B_{12}, 5-methyl-THF is not converted to THF, which is necessary for DNA synthesis, and homocysteine accumulates causing cellular stress.[29] B_{12} is also essential for the conversion of methylmalonyl-CoA mutase to succinyl CoA.[30] During this conversion adenosylcobalamin is the coenzyme to methylmalonyl-CoA mutase in the conversion of methylmalonyl-CoA to succinyl-CoA.

1.1.3 Dietary uptake of B_{12} in humans

Humans have a complicated uptake system for B_{12} requiring specific transport proteins to get B_{12} from dietary sources to proliferating cells. The uptake pathway of B_{12} is designed to both protect and transport. The first two proteins [haptocorrin (HC) and IF] transport B_{12} from the food into the blood and the last protein directs B_{12} to proliferating cells. Figure 3 shows the dietary uptake pathway of B_{12} and how each transport protein is utilized to bring B_{12} from the diet to the cell.

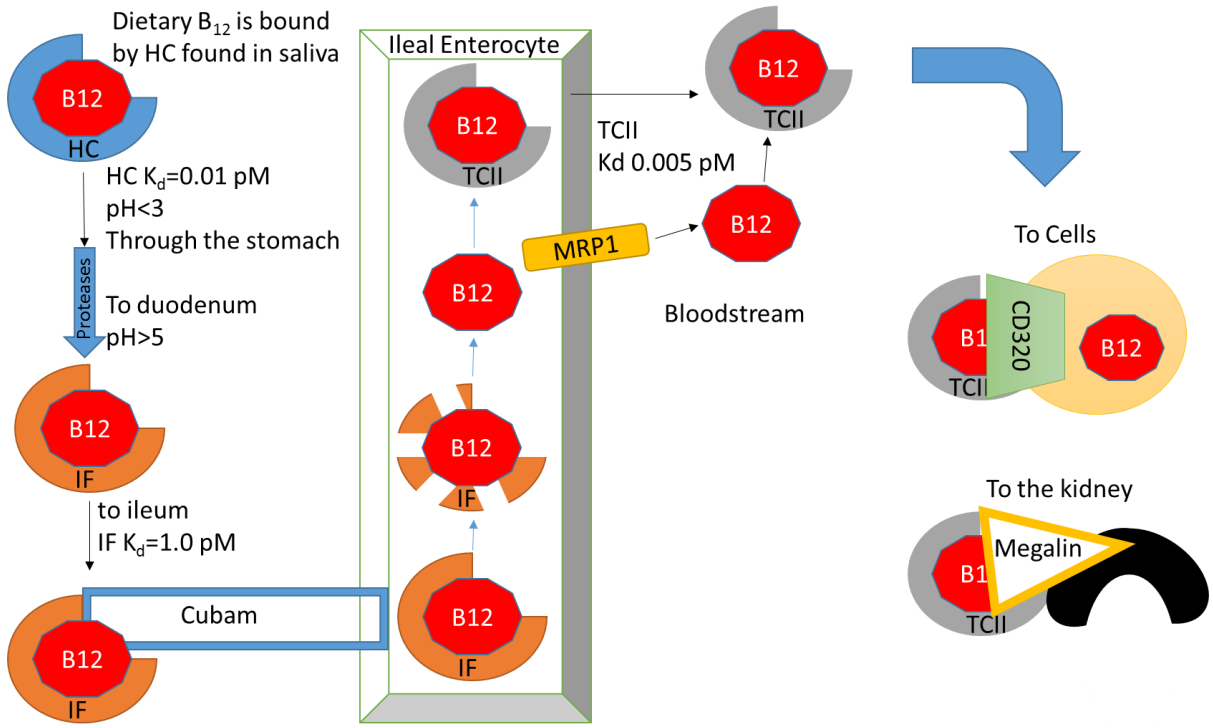


Figure 3. B_{12} dietary uptake pathway. HC: Haptocorrin; MRP: Multidrug resistant protein 1; IF: Intrinsic factor; TCII: Transcobalamin II; CD320: Cluster of differentiation 320.

1.1.4 Transport proteins Involved in B_{12} dietary uptake

There are three binding proteins for the dietary uptake of B_{12}. The binding proteins ensure that B_{12} makes it from food to the cells. These proteins begin chaperoning B_{12} as soon as the food starts to be broken down in the mouth. Protection is necessary since B_{12} is unstable in the low pH environment of the stomach and, hence prone to degradation (much like a peptide would be).

1.1.4.1 Haptocorrin (HC)

The first binding protein involved in B_{12} trafficking is HC, alternative names for which are transcobalamin I or R-binder. HC is the least specific of B_{12}'s binding proteins and recognizes partially degraded fragments of B_{12} along with intact B_{12} with picomolar affinity.[31] HC is a heavily glycosylated ~65 kDa protein and found in both the saliva and blood stream.[32] Figure 3 shows the crystal structure of holo-HC.[33] The HC protein encapsulates B_{12} protecting it from degradation in the stomach. B_{12} is then carried into the duodenum, where the HC is degraded by pancreatic proteases and the B_{12} is picked up by IF.[34]

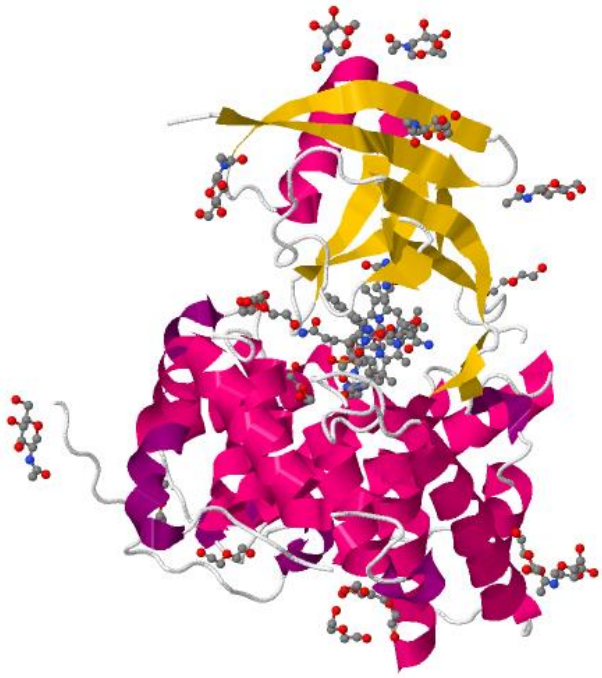


Figure 4. Structure of Holo-HC (pdb: 4KKI), showing the hetero-dimeric nature of the protein with the B_{12} bound at the interface of the dimer.

1.1.4.2 Intrinsic Factor (IF)

The second carrier protein responsible for transporting B_{12} through the intestinal tract and into the blood is IF, a 50 kDa glycoprotein. The crystal structure of IF, shown in Figure 5 as an IF-B_{12} complex, reveals that it is structurally very similar to HC, being composed of two separate domains and binding B_{12} between these domains.[35] IF, unlike HC, is resistant to pancreatic proteases and binds B_{12} in the duodenum with picomolar affinity.[34] IF-B_{12} travels through the intestines and is bound by the cubilin-amnionless (cubam) receptor in the terminal ileum and internalized into the ileal enterocyte.[36] Cathepsin L is responsible for the breakdown of IF in the lysosome and subsequent release of B_{12}.[37]

A lack of IF causes most B_{12} deficiencies and is particularly known as the cause of pernicious anemia.[17] Pernicious anemia is an autoimmune disorder that results in the loss of the gastric parietal cells responsible for producing IF. This disease is progressive and was considered fatal in the 1800s, since it led to an eventual clinical deficiency of B_{12}. Research into a cure for this disease drove the discovery of B_{12}.[18]

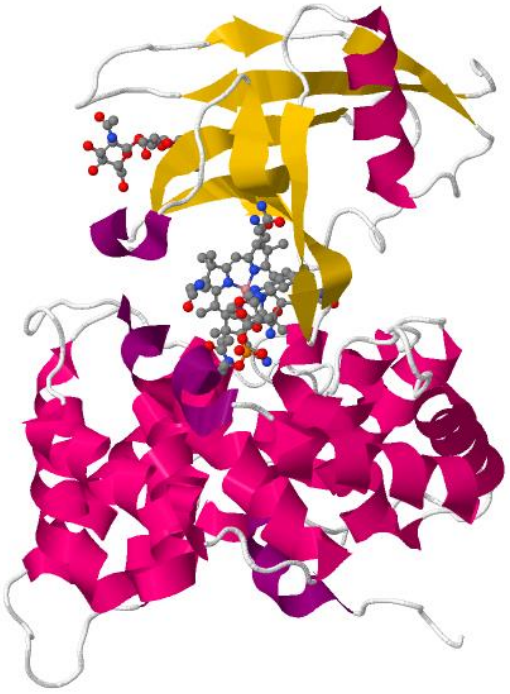


Figure 5. Structure of Holo-IF (pdb: 2PMV), showing the hetero-dimeric nature of the protein with the B_{12} bound at the interface of the dimer.

1.1.4.3 Transcobalamin (TCII)

TCII (shown in Figure 6), the final B_{12} transport protein, is responsible for the delivery of B_{12} to the cells.[38] The TCII-B_{12} complex is delivered to the cells through the CD320 receptor.[39] Unsaturated TCII is found in blood at a concentration of 0.6-1.5 nM in humans, 2 nM in rats, and 20 nM in mice.[40] It is important to consider that rat and mouse models differ in apo-TCII concentration. The TCII concentration is of particular importance when considering B_{12} conjugation as a method to deliver peptides orally. This is because the concentration of B_{12} will have to be over the TCII concentration, or the conjugate may be lost to B_{12} uptake into proliferating cells.

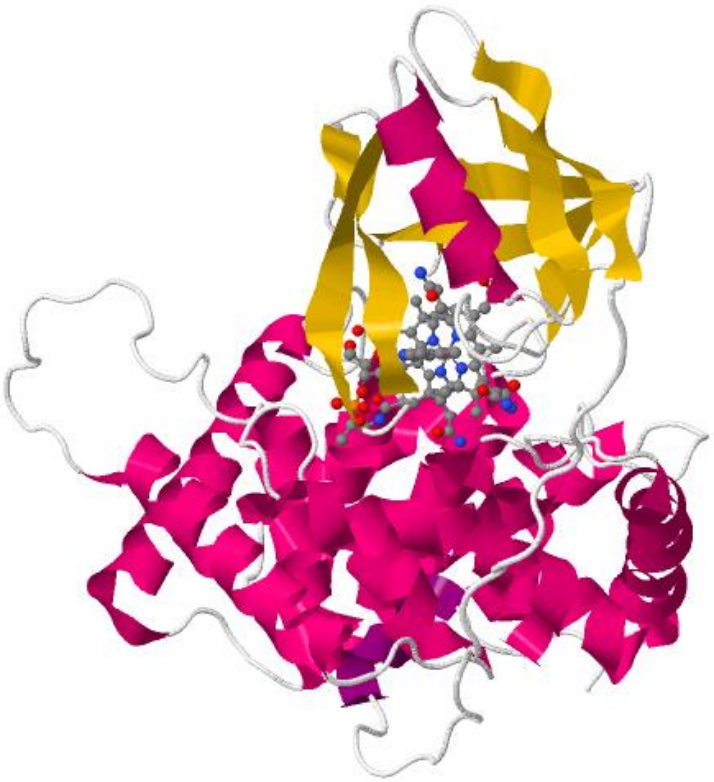


Figure 6. Structure of Holo-TCII (pdb: 2BB5), showing the hetero-dimeric nature of the protein with the B_{12} bound at the interface of the dimer.

1.1.5 Drug development using B_{12} and its dietary pathway

B_{12} and its dietary uptake pathway offer considerable potential in drug development.[41] The first research avenue to be explored was oral delivery of peptides or proteins.[42,43] The hypothesis was that the binding proteins that protect and transfer B_{12} could be manipulated to provide the same protection to peptides and proteins. Another area for research involves the brain uptake of B_{12}. The hydrophilic nature of B_{12} requires a receptor mediated transfer across the blood brain barrier (BBB). The transfer is done through the CD320 receptor and only transports B_{12} in the form of a TCII-B_{12}.

One concern involved with B_{12} conjugation work is the potential for B_{12} to simply act as B_{12}. Will a B_{12} compound act like B_{12} or the drug upon delivery? When B_{12} is in the blood TCII will bind it and deliver it to

proliferating cells so administration over the TCII binding capacity should allow the B_{12} conjugate to behave like the peptide. The second question is whether a B_{12} conjugation will interfere with the function of the drug or molecule. If conjugation cannot be done in a way to maintain function the complex will not work. The obstacles are not unique to B_{12} and can be mitigated with proper controls and dosing.

1.2 Diabetes Mellitus

Diabetes Mellitus is a disease that affects just under 30 million Americans. There are two types of diabetes mellitus, type 1 (T1DM) and type 2 (T2DM).[44] T1DM is an autoimmune disease which destroys the insulin producing beta cells and is typically diagnosed before the age of 30.[45] Since there are no beta cells and insulin cannot be produced the most effective form of treatment is insulin injections. T2DM is caused by a resistance to insulin.[46,47] The β-cells are present and producing insulin in T2DM patients but the body isn't properly utilizing it to clear the glucose from the blood into the cells.[48]

Insulin and glucagon are used in the body to directly control glucose levels. A change in normal glucose levels can be very dangerous to the body so insulin and glucagon work to maintain an homeostasis. Insulin is responsible for glucose entering the cell and glucagon is responsible for the release of glucose stores into the blood.[49] Both hypoglycemia and hyperglycemia can cause serious health issues and death if not properly treated.[50,51]

Insulin is regulated through glucose stimulated insulin secretion (GSIS). An increase in blood glucose levels, causing hyperglycemia, triggers K^+ channels in β-cells to close depolarizing the cell. This causes an influx in Ca^+ and a release of insulin.[52] The insulin then acts to remove the glucose from the blood and deliver it to the cells. In a fasting state, glucose levels are reduced causing hypoglycemia, which is countered through the release of glucose from glycogen stores in the liver. A drop in glucose levels also results in glucagon-induced gluconeogenesis, which helps to restore the glucose levels to normal.[53,54]

1.2.1 Impact of T2DM

T2DM accounts for close to 95% of diabetes patients.[55] Most cases go unnoticed and aren't diagnosed for several years; it is estimated that up to a quarter of Americans with T2DM are undiagnosed.[44] The most common risk factors for T2DM are age and a body mass index (BMI) over 25 kg/m^2.[56] In many cases for people with these risk factors a change in diet can greatly reduce the chances of a diagnosis.[57]

T2DM also has a high cost associated with it. Not only do individuals have to suffer from the ailment of T2DM but they also have to suffer from medical bills and treatment expenditures. Individuals diagnosed with diabetes average medical expenses 2.3 times higher than an individual without diabetes. In 2012 it is estimated that diabetes costs nationwide approached $245 billion.[58] These costs reflect on the total impact of diabetes and just compound to the physical aspects of the disease.[58]

1.2.2 Treatments options for T2DM

Since T2DM patients are able to make insulin, the treatment is different from a T1DM patient. A person with mild T2DM would typically be put on metformin initially. Metformin works to increase insulin sensitivity so that the insulin already produced in the body can work properly.[59] After metformin treatment loses efficacy, typically after the first couple years of treatment, sulfonylureas are added to the treatment regimen.[60] Sulfonylureas act by directly stimulating insulin secretion. This direct action make sulfonylureas very potent but also presents the possibility of hypoglycemia in the case of an overdose. An alternative to sulfonylureas are dipeptidyl peptidase IV (DPP-IV) inhibitors.[61] DPP-IV inhibitors prevent the degradation of incretin hormones which increase insulin levels. This inhibition lets the body release more insulin in a glucose dependent manner.[62] Metformins, sulfonylureas and DPP-IV inhibitors are all available as oral medications, which is why these treatment options are considered first and second line therapies. The third line and fourth line treatments are injectable medications. The third line drugs are incretin mimetics, which act similar to an incretin hormone in the body and increase insulin levels in a glucose

dependent manner.[63] The final treatment option is insulin injection, which is only used after no other treatments work.

1.2.3 Incretin Hormones

A spike in blood glucose levels after ingestion of nutrients causes an increase in insulin levels. This glucose dependent control of insulin is known as the incretin effect. The incretin effect is a response to peptides, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), released in the intestines.[64] Activation of the incretin receptor on pancreatic β-cells results in insulin release dependent on glucose levels.

In T2DM patients the incretin effect is greatly reduced.[65] This is due in large part to the insulinotropic effects of GIP and GLP-1 being either greatly reduced or not present. The insulinotropic effects of GIP are no longer present and GIP shows no effect on insulin secretion when administered even at pharmacological levels.[66,67] GLP-1, however, has been shown to maintain some of its insulinotropic effects albeit at a significantly reduced rate compared to non-T2DM people.[68] The greater effectiveness of GLP-1 to stimulate an insulin release in the presence of T2DM, compared to GIP, has led to the treatments utilizing GLP-1R agonism rather than GIPR agonism.

Of the treatment options for T2DM, only GLP-1 receptor agonist and DPP-IV inhibitors take advantage of the incretin effect. Incretin mimetic therapies are able to promote insulin release in a way that is glucose dependent which means the body is able to regulate how much insulin is released.[69] This is an advantage that minimizes the risk of hypoglycemia. Along with minimized hypoglycemia, incretin therapies have been shown to induce weight loss, promote β-cell regeneration and improve cardiovascular health.[70,71,72]

1.2.3.1 Glucose-dependent insulinotropic peptide

Glucose-dependent insulinotropic peptide (GIP) was the first incretin hormone found. It was initially thought to be only responsible for inhibiting gastric acid secretion but it was later discovered to be responsible for insulin secretion. GIP is released from the intestinal K cells post prandially and activates the GIP receptor (GIPR), a G-protein coupled receptor (GPCR), located in the pancreatic β-cells resulting in an increase in insulin secretion.[73] The half-life of GIP is 2-5 min as a result of degradation from DPP-IV.[74] GIP based T2DM therapies have not had much success because of their relatively weak physiological effects seen especially when compared to GLP-1.[75] There are no current drugs marketed as a GIP agonist, but there is research into the effectiveness of GLP-1 and GIP dual agonism.[6]

1.2.3.2 Glucagon-like peptide-1 (GLP-1)

GLP-1 is an incretin hormone in humans which is primarily responsible for controlling insulin levels in response to a rise in glucose levels in the blood.[76] GLP-1 is produced in the intestinal L cells and is typically released post prandially.[77] DPP-IV quickly degrades GLP-1 in the intestines resulting in rapid clearance ($t_{1/2}$= ~2 minutes). Most humans have a typical fasting plasma level of GLP-1 of 5-10 pmol/L, which can increase upon glucose addition.[64] The GLP-1 receptor (GLP-1R) is located in the pancreatic β-cells as well as the brain, stomach and adipose tissue.[78,79,80,81] Although GLP-1 and GIP are both incretin hormones and promote the release of insulin there are some key differences. GLP-1, unlike GIP, has been shown to inhibit gastric emptying, glucagon secretion and food intake.[82,83,84]

1.2.3.3 Exendin 4 (Ex-4)

Ex-4 is an incretin mimetic, sharing 53% amino acid sequence similarity with GLP-1. Like GLP-1, Ex-4 stimulates the release of insulin through agonism of the GLP-1 receptor (GLP-1R) (EC_{50} 33 pM), effectively lowering blood glucose levels.[85] Unlike GLP-1, Ex-4 is resistant to the enzyme DPP-IV, which rapidly cleaves and inactivates GLP-1 in vivo.[86] Since DPP-IV cleaves any peptide with an alanine or proline

at the second position from the N-terminus, substituting a glycine for the alanine in GLP-1 results in the resistance seen in Ex-4. This resistance allows Ex-4 to have a half-life of 2.4 h compared to <2 min as seen for GLP-1. Such resistance to DPP-IV does not, however, translate to other proteases, and exenatide (synthetic Ex-4) therefore must be administered subcutaneously.

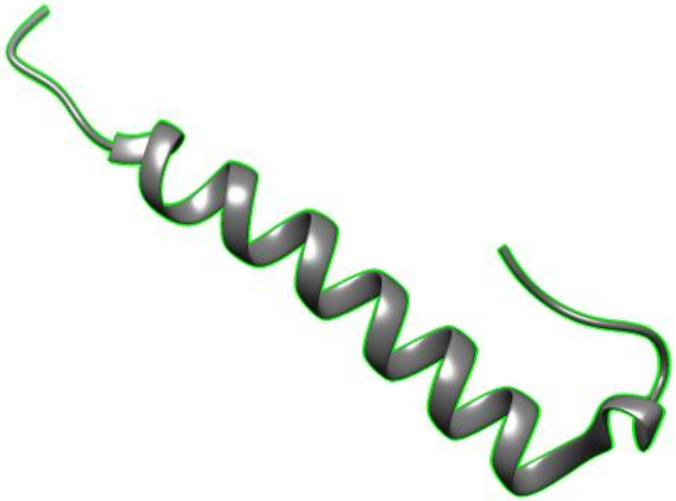


Figure 7. One of the 36 accepted structures of the Ex-4 molecule, pdb1JRJ.[87]

1.2.3.3.1 Discovery of Ex-4

During the late 1980s and early 1990s John Eng, a researcher working for the Veteran Affairs Medical center in the Bronx, was searching for peptides in the glucagon superfamily. His search focused on peptides found in lizards particularly lizards of the *Heloderma* family. A narrow search for peptides found only in *Heloderma suspectum* containing the glucagon superfamily characteristic amino-terminal histidyl structure (His^1) resulted in the discovery of exendin 3, a GLP-1R agonist, in 1990.[88] This exciting result prompted an expansion on the search to include other species within the *Heloderma* family. *Heloderma horridum* was found to contain Ex-4, a potent version of exendin 3, with characteristics similar to those found in GLP-1 in 1992.[89]

1.2.3.4 Glucagon Receptor Family

The glucagon receptor superfamily includes the glucagon receptor (GCGR), GLP-1R, glucagon-like peptide 2 (GLP-2R) and GIPR.[90] These receptors are also part of the larger family of G protein-coupled receptors, and as such contain a specific binding domain. Although the glucagon receptor family and corresponding peptide agonists share many similarities, each receptor maintains a high affinity for only one peptide. However, with careful engineering agonism can be achieved at multiple receptors creating dual and triple agonists of the glucagon receptor superfamily.[91,92]

There are three classes of GPCRs, class A, B, and C, and members of the glucagon receptor superfamily are part of class B.[93,94] Class B GPCRs are part of the secretin family and are activated by endocrine hormones.[95] These receptors contain a 7-transmembrane helical bundle with an N-terminal extracellular domain of 120 amino acids.[96] Upon agonist binding a GPCR undergoes a conformational change resulting in the active state.[96] The G protein acts as a signal transducer upon activation and begins a signal cascade depending on the subunit and pathway. There are three G protein subunits, α, β, and γ and each subunit can be tied to a number of different pathways with the most well know being G_s, G_q, and G_i.[97] Upon GPCR signaling, *β*-arrestin proteins bind to the GPCR and desensitize the GPCR, ending the signal cascade.[98,99]

Members of the glucagon receptor superfamily activate the G_s pathway resulting in an increase in cyclic adenosine monophosphate (cAMP). The increase in cAMP production activates the protein kinase A (PKA) in the cell. The increase in cAMP and active PKA can be used to follow agonism in receptor assays.[100]

1.2.3.4.1 Pharmaceutical applications of Ex-4

Exenatide, a synthetic Ex-4, was approved as a pharmaceutical as the first incretin mimetic under the name Byetta in 2005.[101] The role of exenatide is to increase insulin production and reduce the need for insulin injections. Typical treatment with exenatide is 5 μg injections twice daily for the first month then

10 µg injections twice daily.[102] The half-life of exenatide is 2.1 hours and it is removed from the blood by glomerular filtration and is subsequently degraded by proteases.[103]

1.2.4 Obesity

Obesity and diabetes are comorbidities and approximately 80-95% of patients with T2DM are obese.[104] However, obesity alone affects significantly more people with estimates reporting over a third of Americans as obese.[105] The recent rise in obesity and the projections of increased prevalence through 2050 along with the risk factors associated with the comorbidity of T2DM mean an effective drug to treat both is essential.[106,107] The most effective treatment of obesity to date is bariatric surgery, which is an expensive and invasive to the patient.[108] The invasive nature, severity and cost of bariatric surgery limit the procedure's reach.[109] However the alternative drugs currently on the market are only capable of 5-10% weight reduction.[110] The effectiveness of weight-loss treatment, especially in single drug treatments, is often mitigated by counter-regulatory compensation effects.[111]

1.2.4.1 Peptide Tyrosine Tyrosine (PYY)

Peptide tyrosine tyrosine (PYY) is a peptide is an appetite regulating peptide found in the neuropeptide Y hormone family.[112] It is secreted from the enteroendocrine L-cells of the intestines postprandially. There are two active forms of PYY, PYY(1-36) and PYY(3-36). The initial and short lived PYY is PYY(1-36), which has a half-life only a couple of minutes in blood. The short half-life is due to dipeptidyl-peptidase IV (DPP-IV) which cleaves PYY(1-36) after the second N-terminal amino acid forming PYY(3-36), which has a half-life of just under 16 minutes.[113] PYY(1-36) is an appetite stimulant which upon truncation becomes PYY(3-36), an appetite suppressant, and the major circulating form of PYY.

1.2.4.1.1 PYY(3-36) function

PYY(1-36) is cleaved by DPP-IV, which produces PYY(3-36) and eliminates any function at the neuropeptide Y1 receptor (NPY1R). PYY(3-36) acts only at the neuropeptide Y2 receptor (NPY2R) which is

responsible for appetite suppressing effects. Administration of PYY(3-36) peripherally has been shown to reduce food intake in both healthy and obese rodents.[114] These results have laid a promising foundation for research into PYY(3-36) as an anti-obesity therapy.

1.2.4.1.2 Neuropeptide Y receptors (NPYR)

The neuropeptide Y (NPY) receptor family is composed of four receptors (Y1, Y2, Y4 and Y5). Y1 and Y5 receptors are appetite stimulating and Y2 and Y4 are appetite suppressing.[115] The NPY receptors are activated by the peptides NPY, pancreatic polypeptide, PYY(1-36), and PYY(3-36). The NPY receptors are class A GPCRs, which generally activate either G_i or G_q pathways. The G_q protein activates a cascade which raises Ca^{2+} levels which can be followed through in vitro assays using Fura-2, a Ca^{2+} binding dye.[116] The G_i pathway causes a decrease in cAMP levels, which can be difficult to detect so a promiscuous G_q protein is often introduced to produce a positive response.[117]

1.2.4.1.3 PYY(3-36) pharmaceutical applications

PYY(3-36) has potential as a pharmacological peptide for appetite control and obesity. The first research to highlight the role of PYY(3-36) was published by Batterham in 2002, which showed food intake reductions of up to 30%.[112] Since that paper there has been no shortage of research invested in finding a drug involving PYY(3-36), however no drugs have come to market. This lack of clinical translation is due to two characteristics of PYY(3-36): the first is the nausea associated with administration of the peptide and the second and most important factor is that the body is able to overcome its the appetite suppressing abilities after a couple of days of treatment.[118,119]

Therapy involving PYY(3-36) has to be approached from more than one direction to compensate for the body's ability to overcome the PYY(3-36) signaling. This can be approached through a combination therapy involving multiple peptides or through a single peptide targeting multiple targets with the ability to suppress appetite.

1.2.4.2 Combination therapy for diabetes and obesity treatment

Bariatric surgery has been around for decades and is established as a very effective way to treat obesity. Recently it has been realized that bariatric surgery is also an effective way to treat T2DM.[120] Although the exact mechanisms which make bariatric surgery so effective are still relatively unknown, it is known that both GLP-1 and PYY(3-36) levels are considerably higher after surgery.[121] Replication of the effect caused on hormones after bariatric surgery through medication would provide a great tool for treating both obesity and diabetes.

1.2.4.3 Summary

Diabetes is a disease that effects almost a tenth of all Americans, and though it is mostly controlled with proper medical care there is still room for improvement. Many treatment options for more severe cases of diabetes come with unpleasant side effects or an invasive method of administration. Changes in administration from injection to oral would do a great deal to improve patient compliance with third line medications. Changes in hydrophobicity could alter brain uptake and minimize unwanted side effects. The nature of B_{12} makes it a suitable candidate to alter both of these characteristics. This gives a B_{12}-Ex-4 conjugate the potential to be an oral diabetes medication with limited side effects.

The comorbidity of obesity and diabetes is particularly concerning and although there are methods to control diabetes there are far fewer options to control obesity. Although Ex-4 isn't approved for weight loss it is one of the side effects to the treatment. Enhancements to the appetite suppressing ability of Ex-4 without diminishing its ability to control glucose levels could lead to a diabetes/obesity treatment combined in one drug. PYY is a natural peptide designed to suppress appetite in humans, however the short half-life of PYY makes it unsuitable as a pharmaceutical. Tailoring Ex-4 to activate both the GLP-1R and NPY2R by constructing a hybrid Ex-4/PYY peptide could lead to a single peptide with dual agonism at both GLP-1R and NPY2R.

Modifications to Ex-4 either through B_{12} conjugation or sequence manipulation could provide important improvements to the pharmacokinetics and pharmacodynamics of Ex-4. Success in this work could open the doors to both B_{12} chemistry and Ex-4 chemistry. B_{12} modification to Ex-4 could provide a method to mitigate side effects and be used as a tool in pharmaceutical peptide chemistry to alter brain uptake. A coagonist of GLP-1R and NPY2R drug would provide a method for treating both diabetes and obesity, simultaneously.

1.3 References

1. Kennedy, D. O., B Vitamins and the Brain: Mechanisms, Dose and Efficacy—A Review. *Nutrients* **2016,** *8* (2), 68.

2. Stubbe, J., Binding site revealed of nature's most beautiful cofactor. *Science* **1994,** *266* (5191), 1663-1664.

3. Giedyk, M.; Goliszewska, K.; Gryko, D., Vitamin B12 catalysed reactions. *Chemical Society Reviews* **2015,** *44* (11), 3391-3404.

4. Abeles, R. H.; Dolphin, D., The vitamin B12 coenzyme. *Accounts of Chemical Research* **1976,** *9* (3), 114-120.

5. Nielsen, M. J.; Rasmussen, M. R.; Andersen, C. B.; Nexo, E.; Moestrup, S. K., Vitamin B12 transport from food to the body's cells--a sophisticated, multistep pathway. *Nat Rev Gastroenterol Hepatol* **2012,** *9* (6), 345-354.

6. Finan, B.; Müller, T. D.; Clemmensen, C.; Perez-Tilve, D.; DiMarchi, R. D.; Tschöp, M. H., Reappraisal of GIP Pharmacology for Metabolic Diseases. *Trends in Molecular Medicine 22* (5), 359-376.

7. Brey, R.; Banner, C.; Wolf, J., Cloning of multiple genes involved with cobalamin (Vitamin B12) biosynthesis in Bacillus megaterium. *Journal of bacteriology* **1986,** *167* (2), 623-630.

8. Crouzet, J.; Cauchois, L.; Blanche, F.; Debussche, L.; Thibaut, D.; Rouyez, M.; Rigault, S.; Mayaux, J.; Cameron, B., Nucleotide sequence of a Pseudomonas denitrificans 5.4-kilobase DNA fragment containing five cob genes and identification of structural genes encoding S-adenosyl-L-methionine: uroporphyrinogen III methyltransferase and cobyrinic acid a, c-diamide synthase. *Journal of bacteriology* **1990,** *172* (10), 5968-5979.

9. Crouzet, J.; Levy-Schil, S.; Cameron, B.; Cauchois, L.; Rigault, S.; Rouyez, M.; Blanche, F.; Debussche, L.; Thibaut, D., Nucleotide sequence and genetic analysis of a 13.1-kilobase-pair Pseudomonas denitrificans DNA fragment containing five cob genes and identification of structural genes encoding Cob (I) alamin adenosyltransferase, cobyric acid synthase, and bifunctional cobinamide kinase-cobinamide phosphate guanylyltransferase. *Journal of bacteriology* **1991,** *173* (19), 6074-6087.

10. Roth, J. R.; Lawrence, J. G.; Rubenfield, M.; Kieffer-Higgins, S.; Church, G. M., Characterization of the cobalamin (vitamin B12) biosynthetic genes of Salmonella typhimurium. *Journal of bacteriology* **1993,** *175* (11), 3303-3316.

11. Petrus, A. K.; Fairchild, T. J.; Doyle, R. P., Traveling the Vitamin B12 Pathway: Oral Delivery of Protein and Peptide Drugs. *Angewandte Chemie International Edition* **2009,** *48* (6), 1022-1028.

12. Herbert, V., Recommended dietary intakes (RDI) of vitamin B-12 in humans. *The American Journal of Clinical Nutrition* **1987,** *45* (4), 671-678.

13. Medicine, I. o., *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. The National Academies Press: Washington, DC, 1998; p 592.

14. Shemin, D.; Bray, R. C., The biosynthesis of the corrin structure of vitamin B12*. *Annals of the New York Academy of Sciences* **1964,** *112* (2), 615-621.

15. Wuerges, J.; Geremia, S.; Randaccio, L., Structural study on ligand specificity of human vitamin B12 transporters. *Biochemical Journal* **2007,** *403* (Pt 3), 431-440.

16. Tran, M. T. Q.; Stürup, S.; Lambert, I. H.; Gammelgaard, B.; Furger, E.; Alberto, R., Cellular uptake of metallated cobalamins. *Metallomics* **2016,** *8* (3), 298-304.

17. Bizzaro, N.; Antico, A., Diagnosis and classification of pernicious anemia. *Autoimmunity Reviews* **2014,** *13* (4–5), 565-568.

18. Sinclair, L., Recognizing, treating and understanding pernicious anaemia. *Journal of the Royal Society of Medicine* **2008,** *101* (5), 262-264.

19. Combe, J. S., History of a case of anaemia. *Transactions of the Medico-Chirurgical Society of Edinburgh* **1824,** *1*, 194-204.

20. Whipple, G. H.; Robscheit-Robbins, F. S., THe regeneration of blood in anemia. *Journal of the American Medical Association* **1925,** *85* (1), 36-37.

21. Minot, G. R.; Murphy, W. P., Treatment of pernicious anemia by a special diet. *The Yale Journal of Biology and Medicine* **1926,** *74* (5), 341-353.

22. Okuda, K., Discovery of vitamin B12 in the liver and its absorption factor in the stomach: A historical review. *Journal of Gastroenterology and Hepatology* **1999,** *14* (4), 301-308.

23. Castle, W. B., Observations on the etiologic relationship of achylia gastrica to pernicious anemia. *The American Journal of the Medical Sciences* **1929,** *178* (6), 748-763.

24. Castle, W. B.; Townsend, W. C.; HEATH, C. W., Observations on the etiologic relationship of achylia gastrica to pernicious anemia.: iii. the nature of the reaction between normal human gastric juice and beef muscle leading to clinical improvement and increased blood formation similar to the effect of liver feeding. *The American Journal of the Medical Sciences* **1930,** *180* (3), 305-335.

25. Scott, J. M.; Molloy, A. M., The Discovery of Vitamin B12. *Annals of Nutrition and Metabolism* **2012,** *61* (3), 239-245.

26. Rickes, E. L.; Brink, N. G.; Koniuszy, F. R.; Wood, T. R.; Folkers, K., Crystalline vitamin B12. *American Association for the Advancement of Science. Science* **1948,** *107*, 396-397.

27. Smith, E. L., Purification of anti-pernicious anaemia factors from liver. *Nature* **1948,** *161* (4095), 638-638.

28. D. C. Hogdkin; FRS; Kamper, J.; Mackay, M.; Pickworth, J.; Trueblood, K.; White, J., Structure of vitamin B12. *Nature* **1956,** *178*, 64-66.

29. Guéant, J.-L.; Caillerez-Fofou, M.; Battaglia-Hsu, S.; Alberto, J.-M.; Freund, J.-N.; Dulluc, I.; Adjalla, C.; Maury, F.; Merle, C.; Nicolas, J.-P.; Namour, F.; Daval, J.-L., Molecular and cellular effects of vitamin B12 in brain, myocardium and liver through its role as co-factor of methionine synthase. *Biochimie* **2013,** *95* (5), 1033-1040.

30. Takahashi-Iñiguez, T.; García-Hernandez, E.; Arreguín-Espinosa, R.; Flores, M. E., Role of vitamin B12 on methylmalonyl-CoA mutase activity. *Journal of Zhejiang University SCIENCE B* **2012,** *13* (6), 423-437.

31. Fedosov, S. N.; Fedosova, N. U.; Kräutler, B.; Nexø, E.; Petersen, T. E., Mechanisms of Discrimination between Cobalamins and Their Natural Analogues during Their Binding to the Specific B12-Transporting Proteins. *Biochemistry* **2007,** *46* (21), 6446-6458.

32. Furger, E.; Fedosov, S. N.; Launholt Lildballe, D.; Waibel, R.; Schibli, R.; Nexo, E.; Fischer, E., Comparison of Recombinant Human Haptocorrin Expressed in Human Embryonic Kidney Cells and Native Haptocorrin. *PLoS ONE* **2012,** *7* (5), e37421.

33. Furger, E.; Frei, D. C.; Schibli, R.; Fischer, E.; Prota, A. E., Structural Basis for Universal Corrinoid Recognition by the Cobalamin Transport Protein Haptocorrin. *The Journal of Biological Chemistry* **2013,** *288* (35), 25466-25476.

34. Allen, R. H.; Seetharam, B.; Podell, E.; Alpers, D. H., Effect of Proteolytic Enzymes on the Binding of Cobalamin to R Protein and Intrinsic Factor: In Vitro Evidence That A Failure To Partially Degrader Protein Is Responsible For Cobalamin Malabsorption In Pancreatic Insufficiency. *Journal of Clinical Investigation* **1978,** *61* (1), 47-54.

35. (a) Alpers, D. H.; Russell-Jones, G., Gastric intrinsic factor: The gastric and small intestinal stages of cobalamin absorption. A personal journey. *Biochimie* **2013,** *95* (5), 989-994; (b) Mathews, F. S.; Gordon, M. M.; Chen, Z.; Rajashankar, K. R.; Ealick, S. E.; Alpers, D. H.; Sukumar, N., Crystal structure of human intrinsic factor: cobalamin complex at 2.6-A resolution. *Proceedings of the National Academy of Sciences of the United States of America* **2007,** *104* (44), 17311-17316.

36. Kozyraki, R.; Fyfe, J.; Kristiansen, M.; Gerdes, C.; Jacobsen, C.; Cui, S.; Christensen, E. I.; Aminoff, M.; de la Chapelle, A.; Krahe, R., The intrinsic factor–vitamin B12 receptor, cubilin, is a high-affinity apolipoprotein AI receptor facilitating endocytosis of high-density lipoprotein. *Nature medicine* **1999,** *5* (6), 656-661.

37. Gordon, M. M.; Howard, T.; Becich, M. J.; Alpers, D. H., Cathepsin L mediates intracellular ileal digestion of gastric intrinsic factor. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **1995,** *268* (1), G33-G40.

38. Wuerges, J.; Garau, G.; Geremia, S.; Fedosov, S. N.; Petersen, T. E.; Randaccio, L., Structural basis for mammalian vitamin B(12) transport by transcobalamin. *Proceedings of the National Academy of Sciences of the United States of America* **2006,** *103* (12), 4386-4391.

39. Lai, S.-C.; Nakayama, Y.; Sequeira, J. M.; Wlodarczyk, B. J.; Cabrera, R. M.; Finnell, R. H.; Bottiglieri, T.; Quadros, E. V., The transcobalamin receptor knockout mouse: a model for vitamin B12 deficiency in the central nervous system. *The FASEB Journal* **2013,** *27* (6), 2468-2475.

40. Workinger, J. L.; Doyle, R. P., Vitamin B_{12}: advances and insights; Vitamin B_{12} and Drug Development. *Science Publishers* **2016**.

41. Clardy, S. M.; Allis, D. G.; Fairchild, T. J.; Doyle, R. P., Vitamin B12 in drug delivery: breaking through the barriers to a B12 bioconjugate pharmaceutical. *Expert opinion on drug delivery* **2011,** *8* (1), 127-140.

42. Francis, M. F.; Cristea, M.; Winnik, F. M., Exploiting the vitamin B12 pathway to enhance oral drug delivery via polymeric micelles. *Biomacromolecules* **2005,** *6* (5), 2462-2467.

43. Petrus, A. K.; Vortherms, A. R.; Fairchild, T. J.; Doyle, R. P., Vitamin B12 as a carrier for the oral delivery of insulin. *ChemMedChem* **2007,** *2* (12), 1717-1721.

44. 2016 National Diabetes Fact Sheet, Centers for Disease Control and Prevention. .

45. Daneman, D., Type 1 diabetes. *The Lancet 367* (9513), 847-858.

46. Dawed, A. Y.; Zhou, K.; Pearson, E. R., Pharmacogenetics in type 2 diabetes: influence on response to oral hypoglycemic agents. *Pharmacogenomics and Personalized Medicine* **2016,** *9*, 17-29.

47. Petrak, F.; Herpertz, S.; Stridde, E.; Pfützner, A., Psychological Insulin Resistance in Type 2 Diabetes Patients Regarding Oral Antidiabetes Treatment, Subcutaneous Insulin Injections, or Inhaled Insulin. *Diabetes technology & therapeutics* **2013,** *15* (8), 702-710.

48. Kahn, S. E., The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* **2003,** *46* (1), 3-19.

49. Habegger, K. M.; Heppner, K. M.; Geary, N.; Bartness, T. J.; DiMarchi, R.; Tschop, M. H., The metabolic actions of glucagon revisited. *Nat Rev Endocrinol* **2010,** *6* (12), 689-697.

50. Zoungas, S.; Patel, A.; Chalmers, J.; de Galan, B. E.; Li, Q.; Billot, L.; Woodward, M.; Ninomiya, T.; Neal, B.; MacMahon, S., Severe hypoglycemia and risks of vascular events and death. *New England Journal of Medicine* **2010,** *363* (15), 1410-1418.

51. Klein, R., Hyperglycemia and microvascular and macrovascular disease in diabetes. *Diabetes care* **1995,** *18* (2), 258-268.

52. Henquin, J. C., Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **2000,** *49* (11), 1751-1760.

53. Ozcan, L.; Wong, Catherine C. L.; Li, G.; Xu, T.; Pajvani, U.; Park, Sung Kyu R.; Wronska, A.; Chen, B.-X.; Marks, Andrew R.; Fukamizu, A.; Backs, J.; Singer, Harold A.; Yates Iii, John R.; Accili, D.; Tabas, I., Calcium Signaling through CaMKII Regulates Hepatic Glucose Production in Fasting and Obesity. *Cell Metabolism* **2012,** *15* (5), 739-751.

54. Herzig, S.; Long, F.; Jhala, U. S.; Hedrick, S.; Quinn, R.; Bauer, A.; Rudolph, D.; Schutz, G.; Yoon, C.; Puigserver, P.; Spiegelman, B.; Montminy, M., CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* **2001,** *413* (6852), 179-183.

55. International Diabetes Federation. Diabetes atlas. International Diabetes Federation, 2015.

56. Eckel, N.; Mühlenbruch, K.; Meidtner, K.; Boeing, H.; Stefan, N.; Schulze, M. B., Characterization of metabolically unhealthy normal-weight individuals: Risk factors and their associations with type 2 diabetes. *Metabolism* **2015,** *64* (8), 862-871.

57. McGinley, S. K.; Armstrong, M. J.; Boulé, N. G.; Sigal, R. J., Effects of exercise training using resistance bands on glycaemic control and strength in type 2 diabetes mellitus: a meta-analysis of randomised controlled trials. *Acta Diabetologica* **2015,** *52* (2), 221-230.

58. Association, A. D., Economic Costs of Diabetes in the U.S. in 2012. *Diabetes Care* **2013**.

59. Kajbaf, F.; De Broe, M. E.; Lalau, J.-D., Therapeutic Concentrations of Metformin: A Systematic Review. *Clinical Pharmacokinetics* **2016,** *55* (4), 439-459.

60. Chan, S. P.; Colagiuri, S., Systematic review and meta-analysis of the efficacy and hypoglycemic safety of gliclazide versus other insulinotropic agents. *Diabetes Research and Clinical Practice* **2015,** *110* (1), 75-81.

61. Eriksson, J. W.; Bodegard, J.; Nathanson, D.; Thuresson, M.; Nyström, T.; Norhammar, A., Sulphonylurea compared to DPP-4 inhibitors in combination with metformin carries increased risk of severe hypoglycemia, cardiovascular events, and all-cause mortality. *Diabetes Research and Clinical Practice* **2016,** *117*, 39-47.

62. Ahren, B., DPP-4 inhibitors. *Best practice & research. Clinical endocrinology & metabolism* **2007,** *21* (4), 517-533.

63. Nauck, M. A.; Baranov, O.; Ritzel, R. A.; Meier, J. J., Do current incretin mimetics exploit the full therapeutic potential inherent in GLP-1 receptor stimulation? *Diabetologia* **2013,** *56* (9), 1878-1883.

64. Baggio, L. L.; Drucker, D. J., Biology of incretins: GLP-1 and GIP. *Gastroenterology* **2007,** *132* (6), 2131-57.

65. Eriksen, M.; Jensen, D. H.; Tribler, S.; Holst, J. J.; Madsbad, S.; Krarup, T., Reduction of insulinotropic properties of GLP-1 and GIP after glucocorticoid-induced insulin resistance. *Diabetologia* **2015,** *58* (5), 920-928.

66. Kim, W.; Egan, J. M., The Role of Incretins in Glucose Homeostasis and Diabetes Treatment. *Pharmacological Reviews* **2008,** *60* (4), 470-512.

67. Vilsbøll, T.; Krarup, T.; Madsbad, S.; Holst, J., Defective amplification of the late phase insulin response to glucose by GIP in obese Type II diabetic patients. *Diabetologia* **2002,** *45* (8), 1111-1119.

68. Kjems, L. L.; Holst, J. J.; Vølund, A.; Madsbad, S., The Influence of GLP-1 on Glucose-Stimulated Insulin Secretion. *Effects on β-Cell Sensitivity in Type 2 and Nondiabetic Subjects* **2003,** *52* (2), 380-386.

69. Nauck, M., Incretin therapies: highlighting common features and differences in the modes of action of glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors. *Diabetes, Obesity and Metabolism* **2016,** *18* (3), 203-216.

70. Tamura, K.; Minami, K.; Kudo, M.; Iemoto, K.; Takahashi, H.; Seino, S., Liraglutide Improves Pancreatic Beta Cell Mass and Function in Alloxan-Induced Diabetic Mice. *PLoS ONE* **2015,** *10* (5), e0126003.

71. Kostev, K.; Rex, J.; Rockel, T.; Heilmaier, C., Effects of selected antidiabetics on weight loss – A retrospective database analysis. *Primary Care Diabetes* **2015,** *9* (1), 74-77.

72. White, W. B.; Baker, W. L., Cardiovascular Effects of Incretin-Based Therapies. *Annual Review of Medicine* **2016,** *67* (1), 245-260.

73. Jorde, R.; Burhol, P. G.; Waldum, H. L.; Schulz, T. B.; Lygren, I.; Florholmen, J., Diurnal variation of plasma gastric inhibitory polypeptide in man. *Scandinavian journal of gastroenterology* **1980,** *15* (5), 617-619.

74. Tatarkiewicz, K.; Hargrove, D. M.; Jodka, C. M.; Gedulin, B. R.; Smith, P. A.; Hoyt, J. A.; Lwin, A.; Collins, L.; Mamedova, L.; Levy, O. E.; D'Souza, L.; Janssen, S.; Srivastava, V.; Ghosh, S. S.; Parkes, D. G., A novel long‐acting glucose‐dependent insulinotropic peptide analogue: enhanced efficacy in normal and diabetic rodents. *Diabetes, Obesity and Metabolism* **2014,** *16* (1), 75-85.

75. Lebovitz, H. E., Incretin-based therapies: facing the realities of benefits versus side effects. *Diabetes technology & therapeutics* **2013,** *15* (11), 909-913.

76. Kreymann, B.; Williams, G.; Ghatei, M. A.; Bloom, S. R., Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet (London, England)* **1987,** *2* (8571), 1300-4.

77. Elliott, R. M.; Morgan, L. M.; Tredger, J. A.; Deacon, S.; Wright, J.; Marks, V., Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. *The Journal of endocrinology* **1993,** *138* (1), 159-166.

78. Hörsch, D.; Göke, R.; Eissele, R.; Michel, B.; Göke, B., Reciprocal cellular distribution of glucagon-like peptide-1 (GLP-1) immunoreactivity and GLP-1 receptor mRNA in pancreatic islets of rat. *Pancreas* **1997,** *14* (3), 290-294.

79. Göke, R.; Larsen, P. J.; Mikkelsen, J. D.; Sheikh, S. P., Distribution of GLP‐1 binding sites in the rat brain: evidence that exendin‐4 is a ligand of brain GLP‐1 binding sites. *European Journal of Neuroscience* **1995,** *7* (11), 2294-2300.

80. Uttenthal, L.; Blazquez, E., Characterization of high‐affinity receptors for truncated glucagon‐like peptide‐1 in rat gastric glands. *FEBS letters* **1990,** *262* (1), 139-141.

81. Valverde, I.; Merida, E.; Delgado, E.; Trapote, M.; Villanueva-Penacarrillo, M., Presence and characterization of glucagon-like peptide-1 (7-36) amide receptors in solubilized membranes of rat adipose tissue. *Endocrinology* **1993,** *132* (1), 75-79.

82. Drucker, D. J.; Nauck, M. A., The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *The Lancet 368* (9548), 1696-1705.

83. Willms, B.; Werner, J.; Holst, J. J.; Orskov, C.; Creutzfeldt, W.; Nauck, M. A., Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7-36) amide in type 2 (noninsulin-dependent) diabetic patients. *The Journal of Clinical Endocrinology & Metabolism* **1996,** *81* (1), 327-332.

84. Gutzwiller, J.-P.; Göke, B.; Drewe, J.; Hildebrand, P.; Ketterer, S.; Handschin, D.; Winterhalder, R.; Conen, D.; Beglinger, C., Glucagon-like peptide-1: a potent regulator of food intake in humans. *Gut* **1999,** *44* (1), 81-86.

85. Thorens, B.; Porret, A.; Bühler, L.; Deng, S.-P.; Morel, P.; Widmann, C., Cloning and Functional Expression of the Human Islet GLP-1 Receptor: Demonstration That Exendin-4 Is an Agonist and Exendin-(9–39) an Antagonist of the Receptor. *Diabetes* **1993,** *42* (11), 1678-1682.

86. Furman, B. L., The development of Byetta (exenatide) from the venom of the Gila monster as an anti-diabetic agent. *Toxicon : official journal of the International Society on Toxinology* **2012,** *59* (4), 464-471.

87. Neidigh, J. W.; Fesinmeyer, R. M.; Prickett, K. S.; Andersen, N. H., Exendin-4 and Glucagon-like-peptide-1: NMR Structural Comparisons in the Solution and Micelle-Associated States. *Biochemistry* **2001,** *40* (44), 13188-13200.

88. Eng, J.; Andrews, P. C.; Kleinman, W. A.; Singh, L.; Raufman, J. P., Purification and structure of exendin-3, a new pancreatic secretagogue isolated from Heloderma horridum venom. *Journal of Biological Chemistry* **1990,** *265* (33), 20259-62.

89. Eng, J., Isolation and Characterization of Exendin-4, an Exendin-3 Analogue from *Heloderma suspetum* Venom. *Journal of Biological Chemistry* **1992,** *267* (11), 4.

90. Brubaker, P. L.; Drucker, D. J., Structure-function of the glucagon receptor family of G protein-coupled receptors: the glucagon, GIP, GLP-1, and GLP-2 receptors. *Receptors & channels* **2002,** *8* (3-4), 179-188.

91. Reimann, F.; Gribble, F. M., G protein-coupled receptors as new therapeutic targets for type 2 diabetes. *Diabetologia* **2016,** *59* (2), 229-233.

92. Finan, B.; Yang, B.; Ottaway, N.; Smiley, D. L.; Ma, T.; Clemmensen, C.; Chabenne, J.; Zhang, L.; Habegger, K. M.; Fischer, K.; Campbell, J. E.; Sandoval, D.; Seeley, R. J.; Bleicher, K.; Uhles, S.; Riboulet, W.; Funk, J.; Hertel, C.; Belli, S.; Sebokova, E.; Conde-Knape, K.; Konkar, A.; Drucker, D. J.; Gelfanov, V.; Pfluger, P. T.; Muller, T. D.; Perez-Tilve, D.; DiMarchi, R. D.; Tschop, M. H., A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. *Nat Med* **2015,** *21* (1), 27-36.

93. Siu, F. Y.; He, M.; de Graaf, C.; Han, G. W.; Yang, D.; Zhang, Z.; Zhou, C.; Xu, Q.; Wacker, D.; Joseph, J. S.; Liu, W.; Lau, J.; Cherezov, V.; Katritch, V.; Wang, M.-W.; Stevens, R. C., Structure of the human glucagon class B G-protein-coupled receptor. *Nature* **2013,** *499* (7459), 444-449.

94. Kirkpatrick, A.; Heo, J.; Abrol, R.; Goddard, W. A., Predicted structure of agonist-bound glucagon-like peptide 1 receptor, a class B G protein-coupled receptor. *Proceedings of the National Academy of Sciences* **2012,** *109* (49), 19988-19993.

95. Lee, S.-M.; Booe, J. M.; Pioszak, A. A., Structural insights into ligand recognition and selectivity for classes A, B, and C GPCRs. *European Journal of Pharmacology* **2015,** *763, Part B*, 196-205.

96. Li, Y.; Sun, J.; Li, D.; Lin, J., Activation and conformational dynamics of a class B G-protein-coupled glucagon receptor. *Physical Chemistry Chemical Physics* **2016,** *18* (18), 12642-12650.

97. Neves, S. R.; Ram, P. T.; Iyengar, R., G Protein Pathways. *Science* **2002,** *296* (5573), 1636-1639.

98. Miller, W. E.; Lefkowitz, R. J., Expanding roles for β-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Current Opinion in Cell Biology* **2001,** *13* (2), 139-145.

99. Smith, J. S.; Rajagopal, S., The β-Arrestins: Multifunctional Regulators of G Protein-coupled Receptors. *Journal of Biological Chemistry* **2016,** *291* (17), 8969-8977.

100. Holz, G. G.; Chepurny, O.; Leech, C.; Roe, M.; Xiaodong, C., *High-throughput FRET assays for fast time-dependent detection of cyclic AMP in pancreatic beta cells*. CRC Press, Taylor & Francis Group: Boca Raton, FL: 2015.

101. Bond, A. In *Exenatide (Byetta) as a novel treatment option for type 2 diabetes mellitus*, Baylor University Medical Center. Proceedings, Baylor University Medical Center: 2006; p 281.

102. Byetta, US Full Prescribing Information.

103. Grossman, S. S., Pathophysiological and Pharmacological Rationale for the Use of Exenatide Once Weekly in Patients with Type 2 Diabetes. *Advances in Therapy* **2014,** *31* (3), 247-263.

104. Astrup, A., Healthy lifestyles in Europe: prevention of obesity and type II diabetes by diet and physical activity. *Public health nutrition* **2001,** *4* (2b), 499-515.

105. Ogden, C. L.; Carroll, M. D.; Kit, B. K.; Flegal, K. M., Prevalence of obesity in the United States, 2009-2010. *NCHS data brief* **2012,** (82), 1-8.

106. Bray, G. A., Medical treatment of obesity: the past, the present and the future. *Best practice & research Clinical gastroenterology* **2014,** *28* (4), 665-684.

107. Solas, M.; Milagro, F. I.; Martínez-Urbistondo, D.; Ramirez, M. J.; Martínez, J. A., Precision Obesity Treatments Including Pharmacogenetic and Nutrigenetic Approaches. *Trends in Pharmacological Sciences* **2016,** *37* (7), 575-593.

108. Münzberg, H.; Laque, A.; Yu, S.; Rezai-Zadeh, K.; Berthoud, H. R., Appetite and body weight regulation after bariatric surgery. *Obesity Reviews* **2015,** *16*, 77-90.

109. Pories, W. J., Bariatric Surgery: Risks and Rewards. *The Journal of Clinical Endocrinology and Metabolism* **2008,** *93* (11 Suppl 1), S89-S96.

110. Daneschvar, H. L.; Aronson, M. D.; Smetana, G. W., FDA-Approved Anti-Obesity Drugs in the United States. *The American Journal of Medicine* **2016,** *129* (8), 879.e1-879.e6.

111. Kakkar, A. K.; Dahiya, N., Drug treatment of obesity: Current status and future prospects. *European journal of internal medicine* **2015,** *26* (2), 89-94.

112. Batterham, R. L.; Cowley, M. A.; Small, C. J.; Herzog, H.; Cohen, M. A.; Dakin, C. L.; Wren, A. M.; Brynes, A. E.; Low, M. J.; Ghatei, M. A.; Cone, R. D.; Bloom, S. R., Gut hormone PYY3-36 physiologically inhibits food intake. *Nature* **2002,** *418* (6898), 650-654.

113. Torang, S.; Bojsen-Moller, K. N.; Svane, M. S.; Hartmann, B.; Rosenkilde, M. M.; Madsbad, S.; Holst, J. J., In vivo and in vitro degradation of peptide YY3-36 to inactive peptide YY3-34 in humans. *American journal of physiology. Regulatory, integrative and comparative physiology* **2016,** *310* (9), R866-874.

114. McGowan, B. M.; Bloom, S. R., Gut hormones regulating appetite and metabolism. *Drug Discovery Today: Therapeutic Strategies* **2007,** *4* (2), 147-151.

115. Zac-Varghese, S.; De Silva, A.; Bloom, S. R., Translational studies on PYY as a novel target in obesity. *Current Opinion in Pharmacology* **2011,** *11* (6), 582-585.

116. Roe, M.; Lemasters, J.; Herman, B., Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell calcium* **1990,** *11* (2-3), 63-73.

117. Xing, H.; Tran, H.-C.; Knapp, T. E.; Negulescu, P. A.; Pollok, B. A., A Fluorescent Reporter Assay for the Detection of Ligands Acting Through GI Protein-Coupled Receptors. *Journal of Receptors and Signal Transduction* **2000,** *20* (4), 189-210.

118. Sloth, B.; Holst, J. J.; Flint, A.; Gregersen, N. T.; Astrup, A., Effects of PYY1–36 and PYY3–36 on appetite, energy intake, energy expenditure, glucose and fat metabolism in obese and lean subjects. *American Journal of Physiology - Endocrinology and Metabolism* **2007,** *292* (4), E1062-E1068.

119. Henry, K. E.; Elfers, C. T.; Burke, R. M.; Chepurny, O. G.; Holz, G. G.; Blevins, J. E.; Roth, C. L.; Doyle, R. P., Vitamin B12 Conjugation of Peptide-YY3–36 Decreases Food Intake Compared to Native Peptide-YY3–36 Upon Subcutaneous Administration in Male Rats. *Endocrinology* **2015,** *0* (0), en.2014-1825.

120. Schauer , P. R.; Bhatt , D. L.; Kirwan , J. P.; Wolski , K.; Brethauer , S. A.; Navaneethan , S. D.; Aminian , A.; Pothier , C. E.; Kim , E. S. H.; Nissen , S. E.; Kashyap , S. R., Bariatric Surgery versus Intensive Medical Therapy for Diabetes — 3-Year Outcomes. *New England Journal of Medicine* **2014,** *370* (21), 2002-2013.

121. Arble, D. M.; Sandoval, D. A.; Seeley, R. J., Mechanisms underlying weight loss and metabolic improvements in rodent models of bariatric surgery. *Diabetologia* **2015,** *58* (2), 211-220.

Chapter 2: Synthesis, characterization, in vitro and in vivo analysis of B_{12} conjugated to Exendin 4

The work reported in this chapter will be submitted to *Diabetes* with the title "Vitamin B12 conjugation of exendin-4 abrogates CNS induced nausea and food intake reduction and reverses hyperglycemic response in the rat" and coauthors: Ron L. Bonaccorso, Clinton L. Elfers, Elizabeth G Mietlicki-Baase, George G. Holz, Matthew Hayes, Christian L. Roth and Robert P. Doyle

The first trials of the in vivo glucose tolerance tests and food intake studies reported in this chapter were completed by me while visiting Seattle Children's Research Institute (photo shown above).

2.1 Introduction

Controlling glucose levels is the goal of diabetes treatment. First and second line drugs, such as metformin and sulfonylureas, are effective, safe, and noninvasive.[1] Third line drugs, though effective and very safe, are administered only through subcutaneous injection, leading to low patient compliance, and often come with unpleasant side effects.[2] Nausea is reported in 50% of the patients taking exenatide, synthetic Ex-4, for example.[3] This side effect is hypothesized to be a result of Ex-4 agonism of GLP-1R in the brain at the arcuate nucleus (AC) of the hypothalmus.[4] GLP-1Rs in the brain are also responsible for the weight reduction associated with GLP-1 mimetics, but the pancreatic receptors are used to control

glucose levels and insulin release.[5,6] If brain uptake can be shut down or reduced, both nausea and weight loss will be decreased.

Ex-4 is able to rapidly cross the blood brain barrier (BBB), due to its lipophilic properties.[7] Ex-4 brain uptake studies have shown that almost 90% of Ex-4 reaches the brain.[8] To prevent BBB passage the lipophilicity of Ex-4 must be altered. One way to do this is through conjugation with a lipophobic molecule. B_{12} transport across membranes is receptor mediated and not capable of passive diffusion which make it a good candidate for conjugation and subsequently preventing or at least inhibiting brain uptake.[9] Only TCII bound B_{12} and not HC bound B_{12}, which composes almost 25% of the total B_{12} binding protein in blood, is able to pass through the blood brain barrier and only a third of serum B_{12} enters the brain.[10,11]

2.2 Design and syntheses of B_{12}-Ex-4 conjugates (7-10)

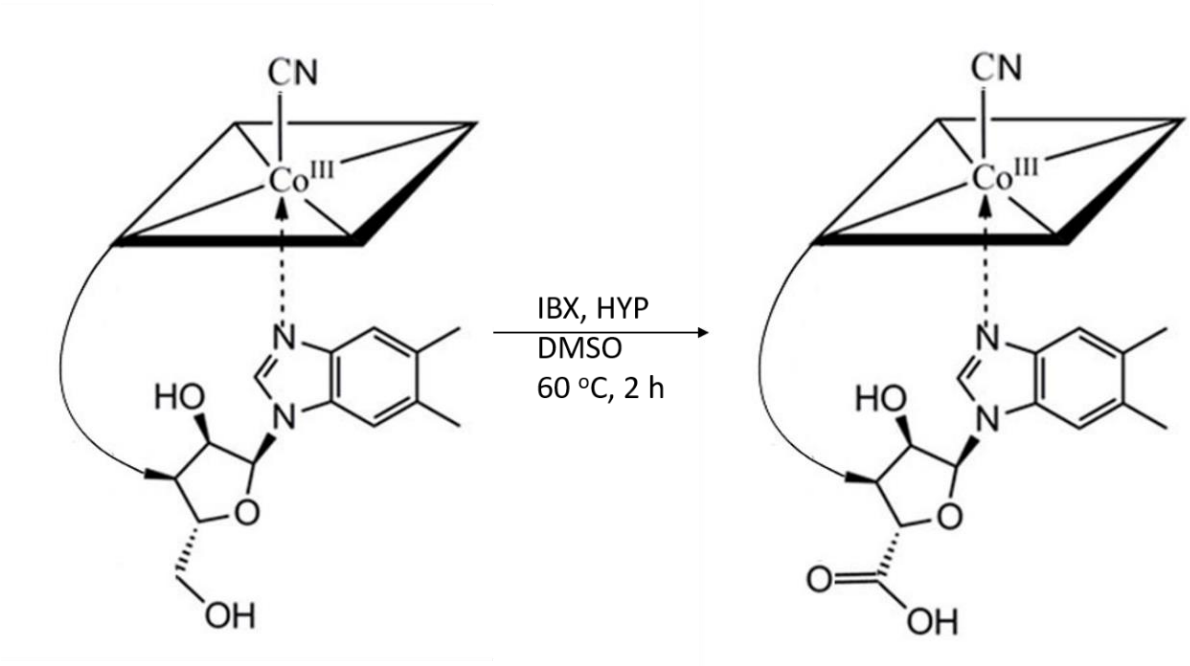
Previously in the Doyle lab, B_{12} had been functionalized with a carboxylic acid through modification of the 5' hydroxyl group of the ribose moiety.[12] Oxidation at this position through use of 2-iodoxybenzoic acid (IBX) allows for further modification and higher yielding peptide conjugations (over 90 percent yield).

2.2.1 Synthesis and characterization of B_{12}-Ex-4 conjugates (7-10)

B_{12}, as cyanocobalamin (**1**), had to be modified in a way that would allow high yielding conjugation between it and Ex-4. This was achieved by modifying the ribose hydroxyl group. B_{12} was initially functionalized to a carboxylic acid.[12] From the carboxylic acid a carbon linker and alkyne functional group could be formed.

2.2.1.1 B_{12} modification of the 5' hydroxyl to a carboxylic acid

This is done by converting the 5' hydroxyl to a carboxylic acid as shown in Figure 1. Modifications are done at this position so that binding to the transfer proteins remains unhampered. This reaction is done using IBX and 2-hydroxypyridine (HYP). The primary alcohol is successfully converted to a carboxylic acid producing B_{12}-CA (**2**) with a 30% yield.



1 **2**

Figure 1. Oxidation of the 5’ hydroxyl group of **1** to a carboxylic acid.

2 was then purified using FPLC and characterized by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-ToF-MS). Figure 2 shows a m/z of **2** of 1343.6 which is consistent with the expected m/z of [M-CN].

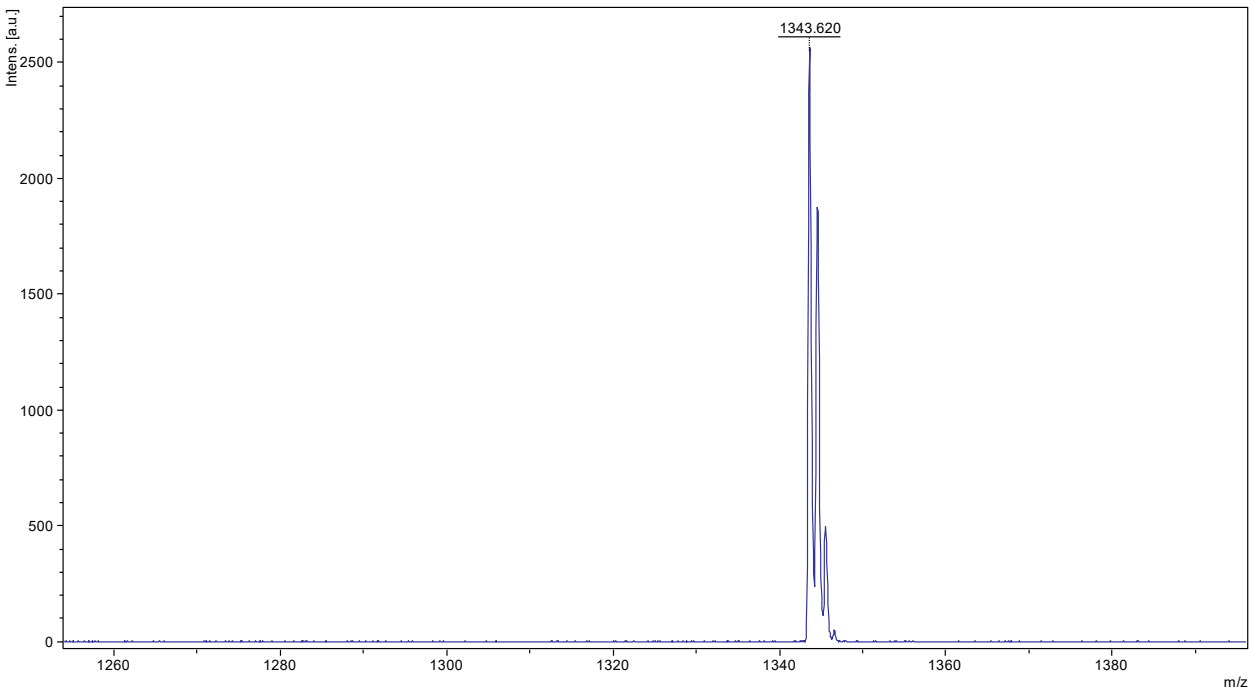
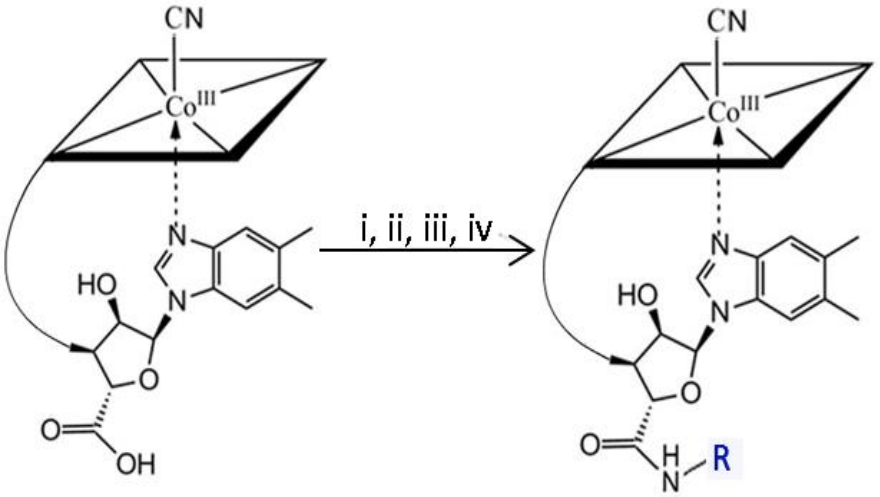


Figure 2. MALDI-ToF MS of **2** expected [M-CN] 1344 m/z, observed 1343.6 m/z.

2.2.1.2 Functionalizing B_{12} through addition of an alkyne via the carboxylic acid

Following the synthesis of **2,** a series of B_{12} molecules containing a terminal alkyne were synthesized. B_{12} was primed for click chemistry by coupling an alkyne-amine to the carboxylic acid of **2**. Four B_{12}-alkyne compounds (**3**-**6**) were synthesized with an alkyl chain containing a terminal alkyne varying from three to six carbons off of the carboxylic acid.[13] Figure 3 shows the scheme used to synthesize the B_{12}-alkyne compounds (**3**-**6**) utilizing 1-ethyl-3-(3-3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt). Successful conversion yielded compounds **2-5** with a yield of >90%.



	R
3	CH_2CCH
4	CH_2CH_2CCH
5	$CH_2CH_2CH_2CCH$
6	$CH_2CH_2CH_2CH_2CCH$

Figure 3. Synthesis of B_{12}-alkyne precursors for B_{12}-Ex-4. (i) EDCl, HOBt, propargyl amine, rt, DMSO, 16 h; (ii) EDCl, HOBt, 1-amino-3-butyne, rt, DMSO, 16 h; (iii) EDCl, HOBt, 1-amino-4-pentyne, rt, DMSO, 16 h; (iv) EDCl, HOBt, 1-amino-5-hexyne, rt, DMSO, 16 h; (v).

3-6 were successfully made and characterized through MALDI-ToF-MS as shown in Figure 4. **3** had an observed mass of [M-CN] 1381.7 m/z with an expected mass of 1382 m/z. **4** had an observed mass of [M-CN] 1393.6 m/z with an expected mass of 1394 m/z. **5** had an observed mass of [M-CN] 1407.7 m/z with an expected mass of 1408 m/z. **6** had an observed mass of [M-CN] 1421.9 m/z with an expected mass of 1422 m/z.

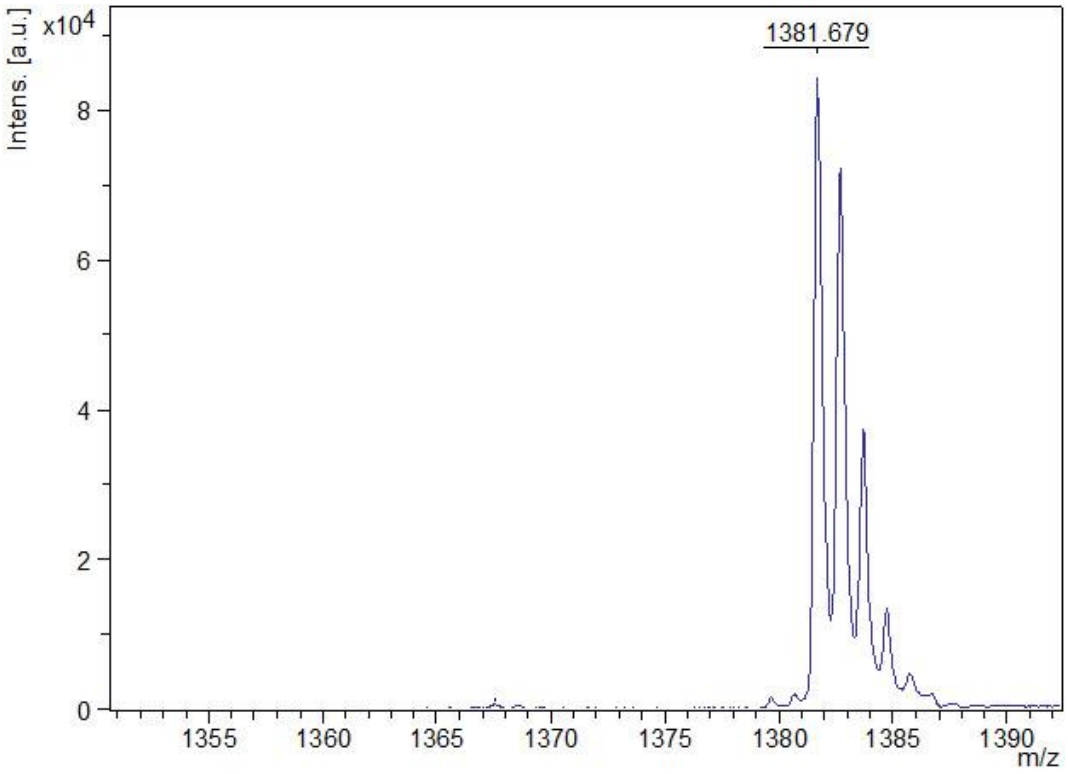


Figure 4A. B_{12}-propyne (**3**) MALDI-ToF MS expected [M-CN] 1380 m/z observed 1381.7 m/z.

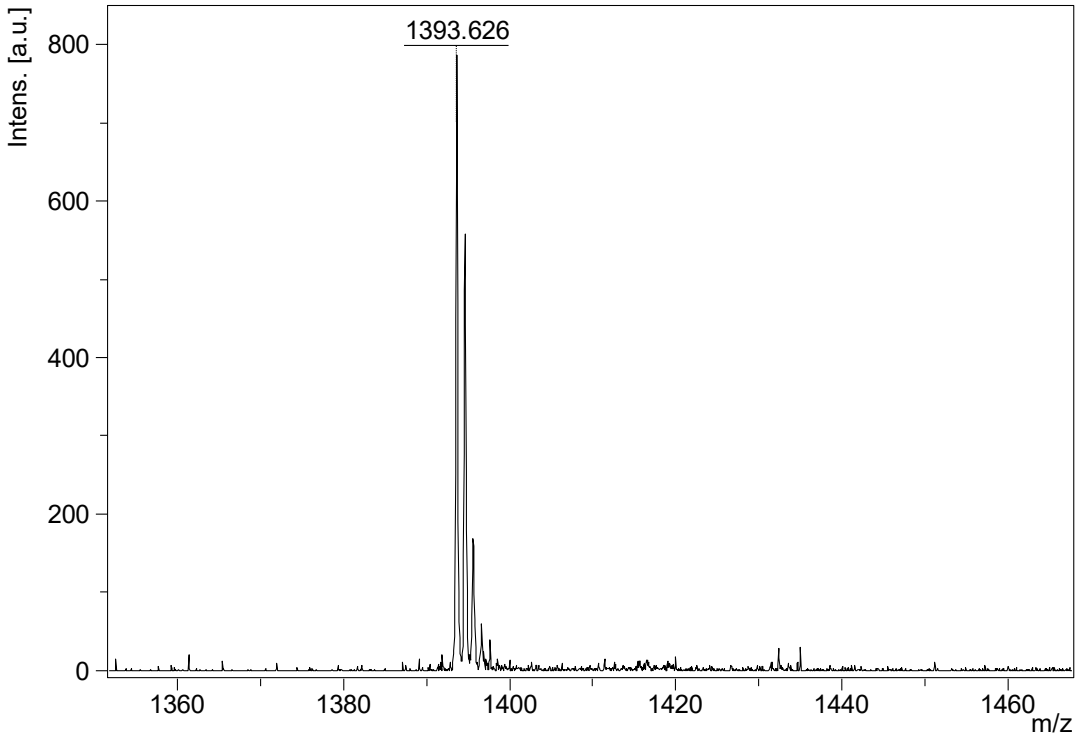


Figure 4B. B_{12}-butyne (**4**) MALDI-ToF MS expected [M-CN] 1394 m/z observed 1393.6 m/z.

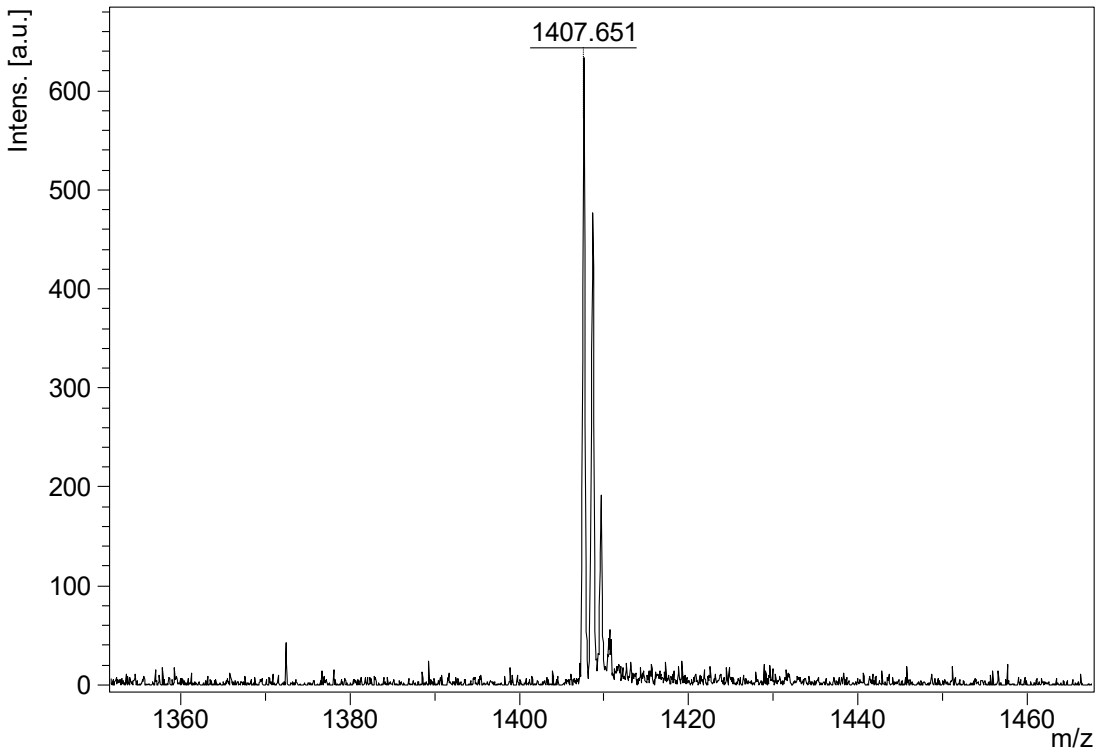


Figure 4C. B_{12}-pentyne (**5**) MALDI-ToF MS expected [M-CN] 1408 m/z observed 1407.7 m/z.

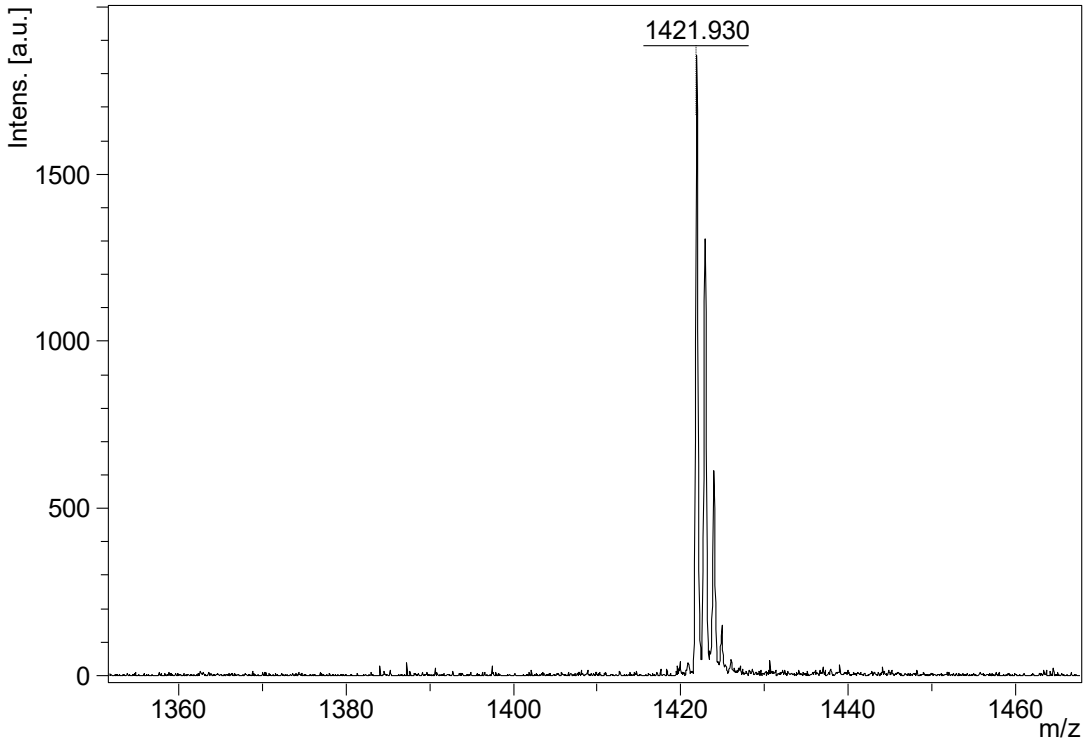
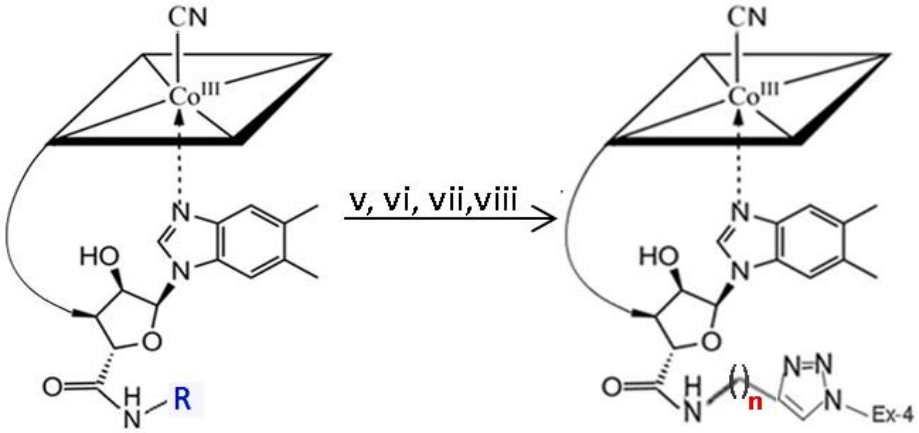


Figure 4D. B_{12}-hexyne (**6**) MALDI-ToF MS expected [M-CN] 1422 m/z observed 1421.9 m/z.

2.2.1.3 B_{12}-Ex-4 conjugates (7-10) synthesized via CuAAC

Compounds **2**-**5** were used to make a series of B_{12}-Ex-4 conjugates with various linker lengths between B_{12} and Ex-4. B_{12} was conjugated to Ex-4 through Sharpless/Huisgen copper-catalyzed azide-alkyne cycloaddition (CuAAC), click chemistry, utilizing copper(II) sulfate ($CuSO_4$) and sodium ascorbate.[14,15,16] Figure 5 shows the schematic for the synthesis of compounds **6**-**9**.



	R
3	CH_2CCH
4	CH_2CH_2CCH
5	$CH_2CH_2CH_2CCH$
6	$CH_2CH_2CH_2CH_2CCH$

	n
7	3
8	4
9	5
10	6

Figure 5. Synthesis of B_{12}-conjugates **6**-**9**. (v) **3**, $CuSO_4$, sodium ascorbate, Ex-4-K12-azido, Water/DMF 4:1, 1 h; (vi) **4**, $CuSO_4$, sodium ascorbate, Ex-4-K12-azido, Water/DMF 4:1, 1 h; (vii) **4**, $CuSO_4$, sodium ascorbate, Ex-4-K12-azido, Water/DMF, 4:1, 1 h; (viii) **6**, $CuSO_4$, sodium ascorbate, Ex-4-K12-azido, Water/DMF; 1 h. >90% yield.

2.3 In vitro optimization of B_{12}-Ex-4 agonism at the GLP-1R

The B_{12}-Ex-4 series was first tested for function at the human GLP-1R in human embryonic kidney (HEK) cells, which had been stably transfected with the receptor. Activation of GLP-1R initiates the G_s cascade of the GPCR raising cAMP and PKA levels in the cell. This activity was monitored through a couple

of different assays using either a bioluminescence assay or FRET to follow the cascade resulting from activation of the GLP-1R.

The first method used to follow GLP-1R agonism involved monitoring a FRET ratio change to follow PKA activity. To follow PKA levels HEK-GLP-1R cells are treated with adenovirus incorporating an A kinase activity reporter (AKAR3).[17] AKAR3 is activated and a FRET change is seen upon phosphorylation by PKA (see Figure 2). This change in FRET is dose dependent and can be used to determine an EC_{50} for GLP-1R agonists.

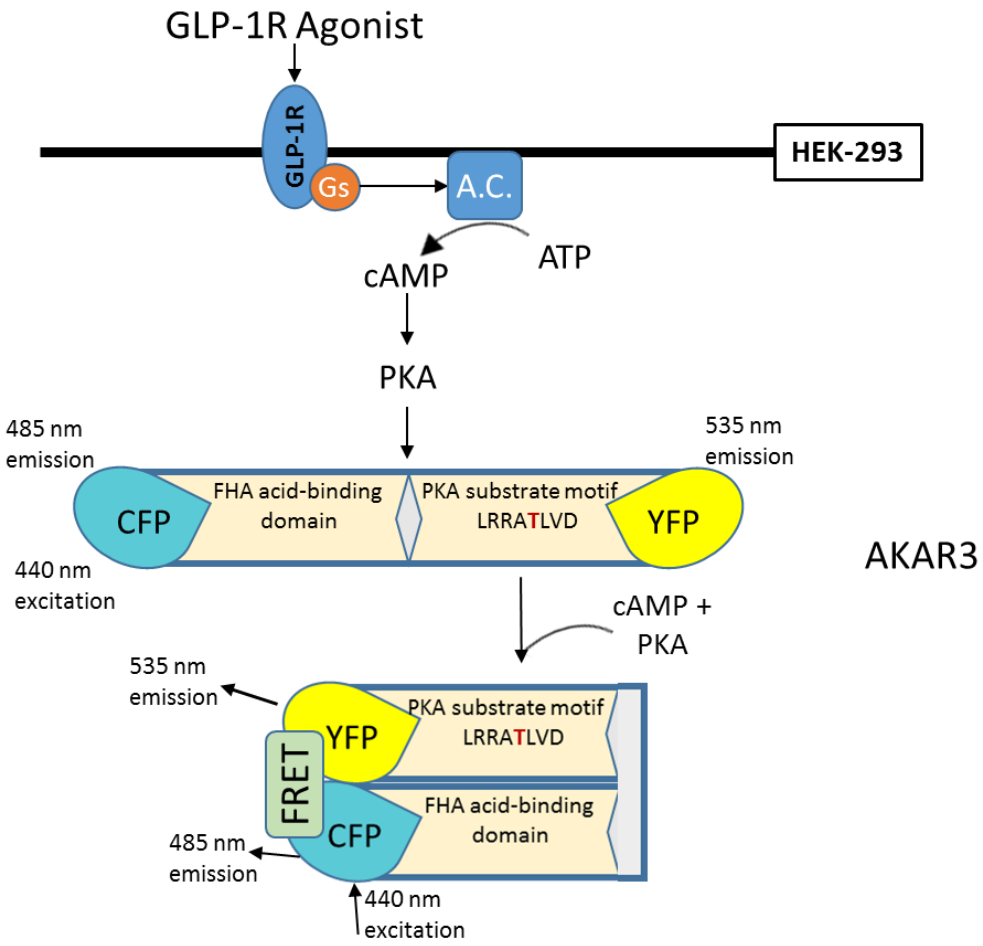


Figure 6. GLP-1R agonist induced GPCR G_s cascade resulting in AKAR3 activation by PKA. GLP-1R: glucagon-like peptide-1 receptor; A.C.: adenylate cyclase; HEK-293: human embryonic kidney cells; ATP: Adenosine

triphosphate; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; YFP: yellow fluorescent protein; CFP: cyan fluorescent protein; FHA: forkhead associated; FRET: fluorescence resonance energy transfer.

B_{12}-Ex-4 activates the G_s pathway of the GPCR upon binding to GLP-1R. The G_s pathway activates adenylate cyclase (A.C.) which converts ATP to cAMP. As the cAMP level rises the enzyme PKA is activated and can then phosphorylate the threonine amino acid of AKAR3. This phosphorylation of AKAR3 results in a conformational change producing a decrease of 485/535 nm emission FRET ratio. This decrease in FRET ratio is dose dependent and can be followed to show EC_{50} values in vitro.

Using AKAR3 to follow FRET ratio change allows for real time monitoring of changes. Figure 8 shows the raw data in real time for a dose response series of **8**. At t=100 s the injection of either saline or **8** at a concentration between 10 pM and 10,000 pM was administered almost immediately resulting in an increase in PKA levels and change in FRET.

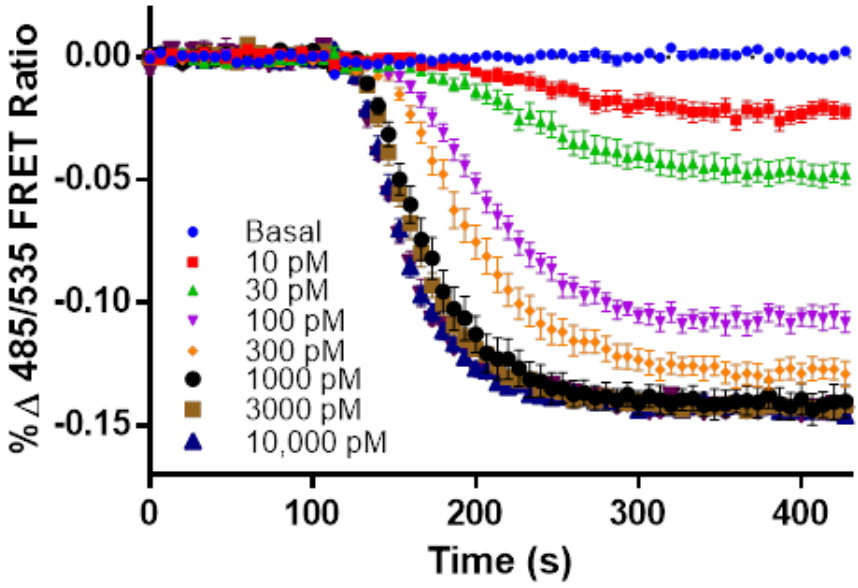


Figure 7. Dose response of PKA phosphorylation of AKAR3 in real time after administration of either saline or **8** of concentrations ranging from 10-10,000 pM.

7-10 were tested for agonism at the GLP-1R. The optimal conjugated Ex-4 would be used as the lead conjugate for further testing. Figure 9 shows the dose response results of each conjugate. The most potent conjugate, **8,** had an EC_{50} of 68 pM followed by **7**, **9**, and **10,** which had EC_{50}'s of 121, 246 and 405 pM, respectively compared to Ex-4 at 26 pM. Ex-4 has been shown to have an EC_{50} of 33 pM by Thorens *et al*. by following cAMP production so this method is consistent with previous literature reports.[18]

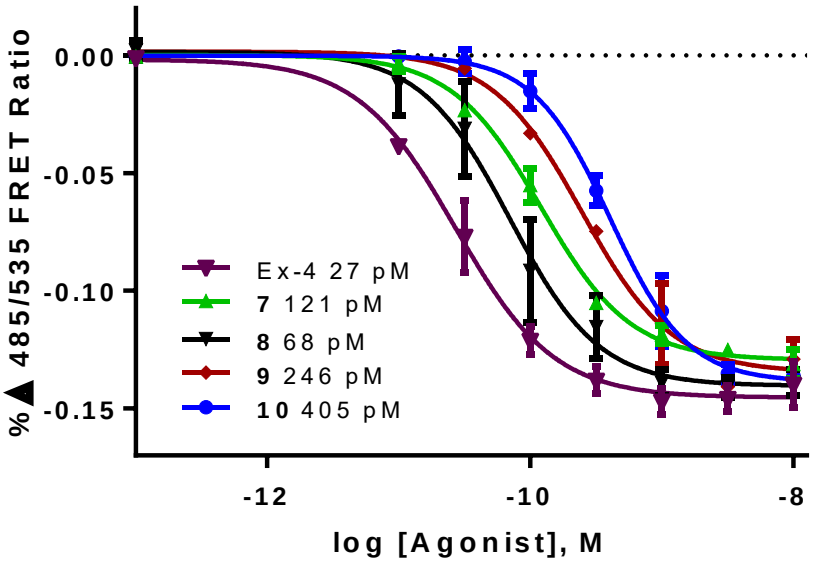


Figure 8. Comparison of Ex-4, **7**, **8**, **9** and **10** EC_{50} curves with EC_{50} values of 27, 121, 68, 246 and 405 pM, respectively.

8 was screened i as the optimal conjugate and was used for all further testing. Conjugation of B_{12} to Ex-4 resulted in a relatively small loss of function and still maintained picomolar function at the GLP-1R. Linker length played a small role in receptor agonism potency as EC_{50} of all of the conjugates were within one log unit, indicating that compounds **7-9** had essentially the same potency, of Ex-4 except for **10**.

2.3.1.1 B_{12}-Ex-4 (8) affinity for B_{12} binding proteins

After synthesis and optimization of the B_{12}-Ex-4 conjugates and analysis of function at the GLP-1R, binding affinities for the B_{12} binding proteins had to be confirmed. For B_{12} to be a viable oral delivery method modifications and conjugations have to be done in a way that won't interfere with the ability of IF to recognize and bind the conjugate. TCII affinity is also important since it is responsible for delivery of B_{12} to cells. Binding of the conjugate to TCII could result in delivery to the CD320 receptor rather than the GLP-1R.

IF and TCII binding of **8** was confirmed by radiometric chase assay using ^{57}Co-labelled B_{12} and compared to free B_{12}, as cyanocobalamin.[19] IF and TCII binding of **8** (Kd values of 6.8 nM and 0.75 nM, respectively) was maintained, albeit reduced from unmodified B_{12} (0.12 nM and 0.098 nM respectively) see Figure 9.

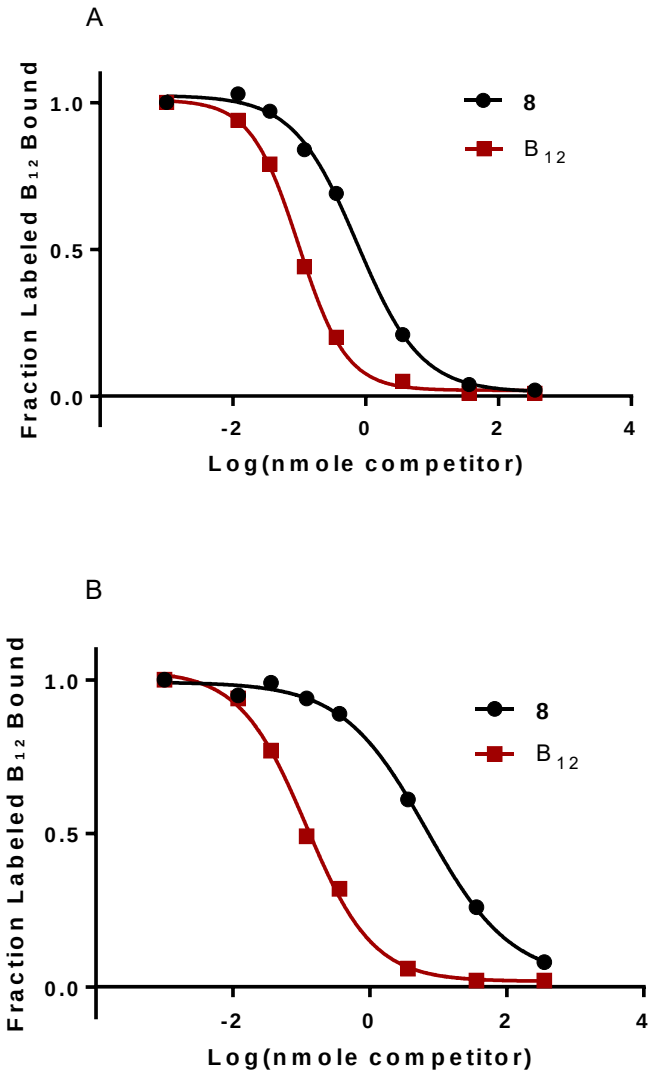


Figure 9. TCII (A) and IF (B) binding of **8** compared to B_{12}.

After establishing binding to the B_{12} binding proteins **8** was tested for function at the GLP-1R while complexed to IF (see Figure 10). Since everywhere B_{12} is in the body it is bound to a binding protein, it is important to confirm that the conjugate could work while complexed to these B_{12} binding proteins. Picomolar agonism of GLP-1R is maintained even after IF complexation indication that **8** could work in the presence of these proteins.

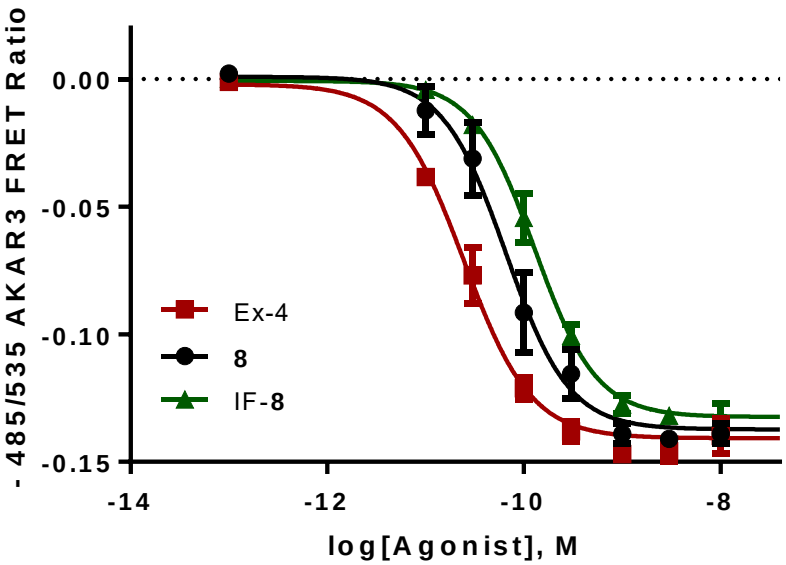


Figure 10. Dose response curve of Ex-4, **8** and IF-**8** yielding EC_{50}s of 27, 68, and 132 pM respectively.

The second method used for assaying GLP-1R agonism followed cAMP levels in the cell. HEK-293 cells stably expressing GLP-1R were first transfected with a rat insulin 1 promoter (RIP1) cAMP response element (CRE) encoding for the enzyme luciferase (Luc) (see Figure 11). The production of the enzyme luciferase is dependent on the cAMP levels after incubation with the GLP-1R agonist. Luciferin is then added to the cells and the amount catalyzed by luciferase can be determined through a bioluminescence readout. B_{12}-Ex-4 and B_{12}-Ex-4 complexed to the binding proteins IF and HC were tested for function and compared to Ex-4. Following cAMP through this RIP-CRE-Luc assay yielded an EC_{50} of 85 pM for Ex-4, 362 pM for B_{12}-Ex-4, 434 for IF-B_{12}-Ex-4 and 426 for HC-B_{12}-Ex-4. Both IF and HC showed similar effect on agonism at GLP-1R.

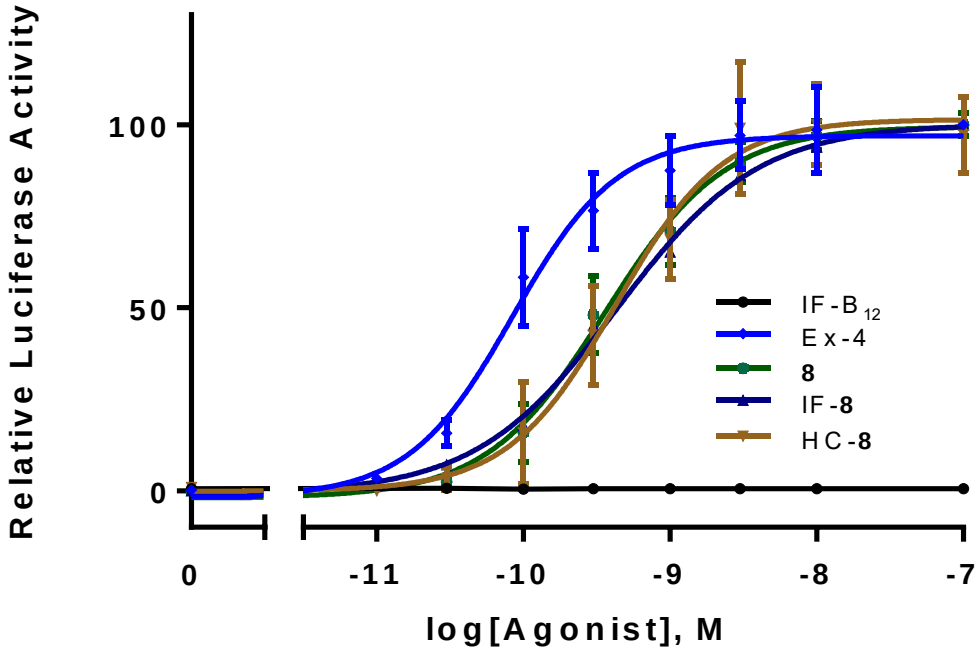


Figure 11. Ex-4, **8**, IF-**8** and HC-**8** function at the GLP-1R monitored through RIP-CRE-Luc.

2.4 In vivo testing of B_{12}-Ex-4 (8) to demonstrate reduced brain uptake compared to Ex-4

2.4.1 Glucose tolerance tests after B_{12}-Ex-4 and Ex-4 administration

Following the body's response to a bolus of glucose is one of the oldest and most widely used tests to determine if a person is diabetic.[20] A person with a normal response will show a blood glucose level below 140 mg/dL whereas a person with T2DM will reach a blood glucose level over 200 mg/dL.[21] There are two different ways to test for glucose tolerance, either with an oral bolus of glucose or an intraperitoneal bolus of glucose followed by an A1C1 reading of the blood at various time intervals.[22,23] Though in both instances the blood glucose levels rise, only through an oral bolus does the incretin effect occur. The body requires glucose to pass through the intestines for the appropriate receptors to be activated. This difference allows for determination of whether an incretin effect and intestinal stimulation is needed for our drug to elicit an effect.

2.4.1.1 Oral glucose tolerance test of 8 compared to Ex-4 and saline

An oral glucose tolerance test (OGTT) was performed on Sprague Dawley rats (n=12) (see Figure 10) to compare the glucose controlling effects of **8** and Ex-4. For testing brain uptake of **8** and Ex-4 the rat model is a particularly effective since activation of the rat's sympathetic nervous system (SNS) causes a characteristic rise in glucose level unlike the sharp decrease seen in mice and humans.[24] Elimination of this glucose spike and an actual decrease in glucose points to a reduction of brain uptake with **8** versus Ex-4, which showed the normal increase in glucose.

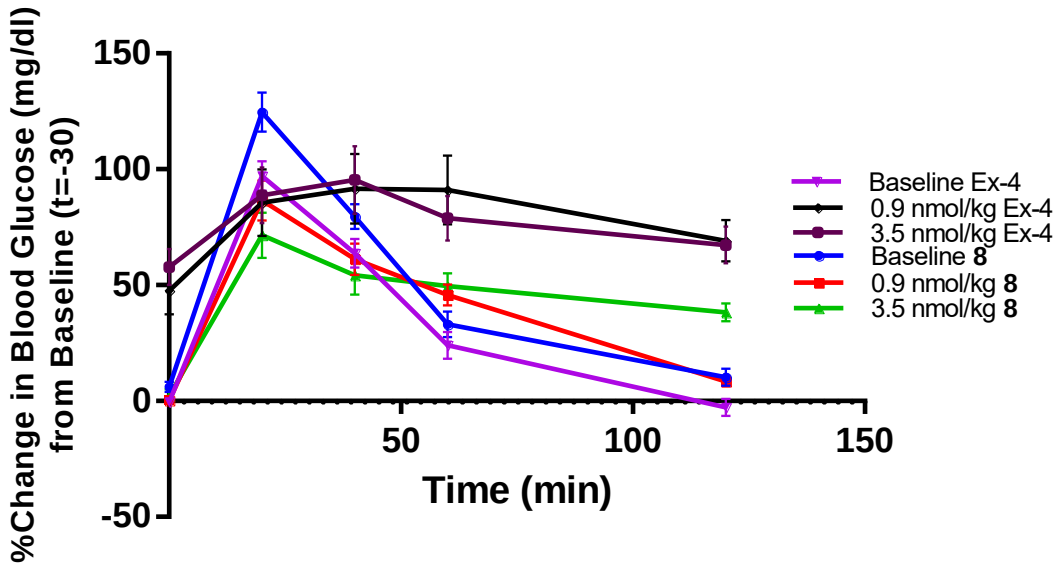


Figure 12. Percent change in blood glucose (mg/dL) in Sprague Dawley rats (n=12) from baseline (t=-30) for vehicle, 0.9 nmol/kg Ex-4, 3.5 nmol/kg Ex-4, 0.9 nmol/kg **8**, and 3.5 nmol/kg **8**.

2.4.2 Food Intake and Body Weight change with 8 administration

A side effect associated with Ex-4 is weight loss, and though generally regarded as a positive it is unknown if the weight loss is a result of the nausea caused by Ex-4 or through some other process in the body.[3] What is known though, is if GLP-1Rs in the brain are not activated the nausea and food intake reduction are not seen.[25,26] If food intake reduction is mitigated, then brain activation of GLP-1Rs on the

AN are not activated. Figure 13 shows that administration of **8** at 0.2, 0.9 or 3.5 nmol/kg doses are ineffective at reducing food intake in rats (n=12).

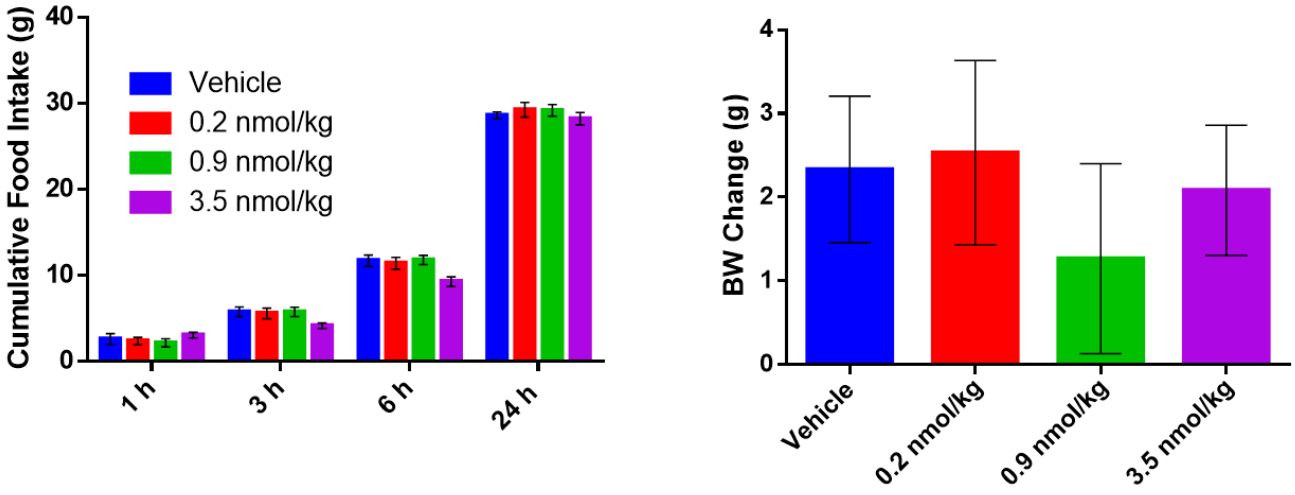


Figure 13. (Left) Food intake and (Right) body weight change over 24 h for either vehicle, 0.2 nmol/kg, 0.9 nmol/kg, or 3.5 nmol/kg of **8** (vehicle, 0.2 nmol/kg, 0.9 nmol/kg, and 3.5 nmol/kg).

Only at the two highest concentrations of **8** was there any change in food intake or body weight. Although this change was not significant the food intake and body weight study was repeated and compared to Ex-4 at a concentration of 0.9 nmol/kg (Figure 14 and Figure 15). Food intake reduction was significant for Ex-4 at 0.9 nmol/kg and significant for **8** but only at a concentration of 3.5 nmol/kg and with almost half the food intake reduction seen for Ex-4. Along with the reduction in food intake was a reduction in body weight. The largest average body weight reduction was from the Ex-4 treatment followed by **8** at 3.5 nmol/kg and then **8** at 0.9 nmol/kg.

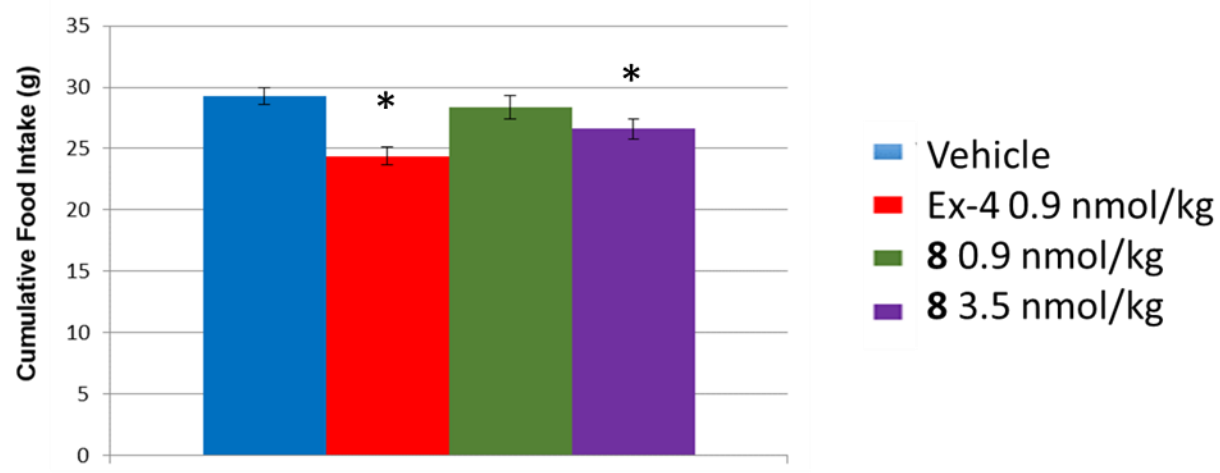


Figure 14. 24 h food intake comparison of Ex-4 at 0.9 nmol/kg to 8 at 0.9 nmol/kg and 3.5 nmol/kg. p=0.00012 for Ex-4 and p=0.023 for 8 at 3.5 nmol/kg when compared to baseline.

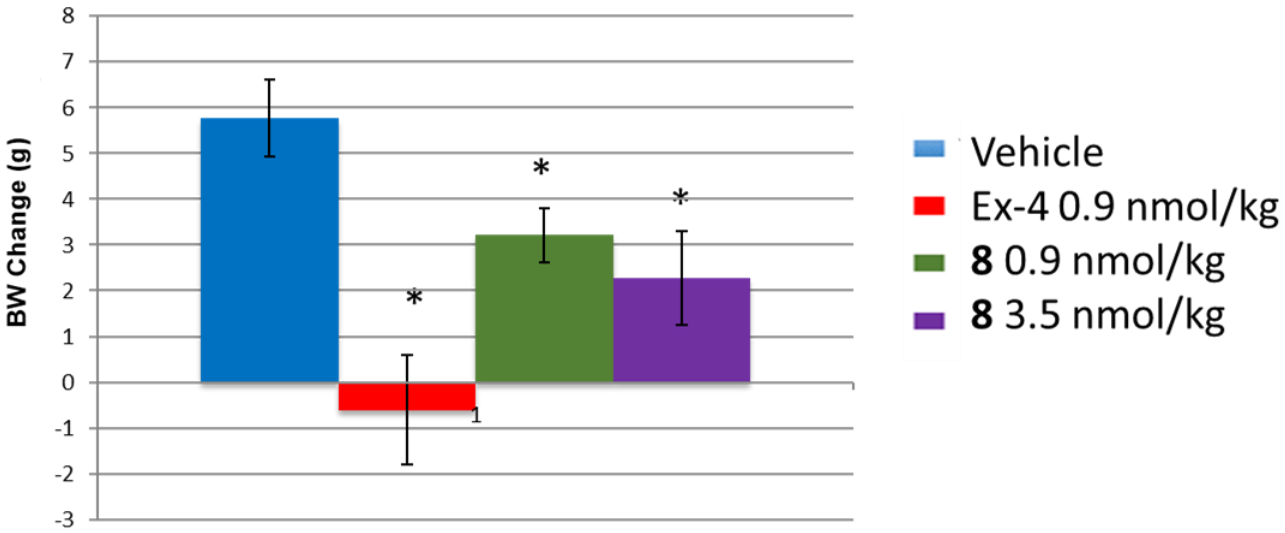


Figure 15. 24 h body weight comparison of Ex-4 at 0.9 nmol/kg to **8** at 0.9 nmol/kg and 3.5 nmol/kg. p≤0.05

2.4.3 PICA studies establishing nausea levels for 8 vs. Ex-4

Measuring nausea in rats as a response to Ex-4 can be done by looking at the way the rats behave. Rats are particularly good model to study nausea on since they are unable to vomit and behave in a predictable way when experiencing nausea.[27] There are a couple of tests that can be used to measure nausea. The first test is conditioned taste aversion and the second is pica, which is when the rat consumes a nonnutritive food such as kaolin,.[28,29]

A pica test was used to follow the level of nausea in the rats by following kaolin consumption. Kaolin intake was followed over 24 hours after administration of 0.9 nmol/kg of Ex-4, 0.9 nmol/kg of **8**, or 3.5 nmol/kg of **8** (see Figure 14). Ex-4 treatment was the only instance of significant increase in kaolin intake when compared to baseline. Concentrations of **8** up to 4x that of Ex-4 still trended to lower nausea as indicated by the reduced kaolin intake. This study is still on-going to increase statistical significance prior to publication (To be submitted to the journal 'Diabetes (IF 12)' with this author as 'first author').

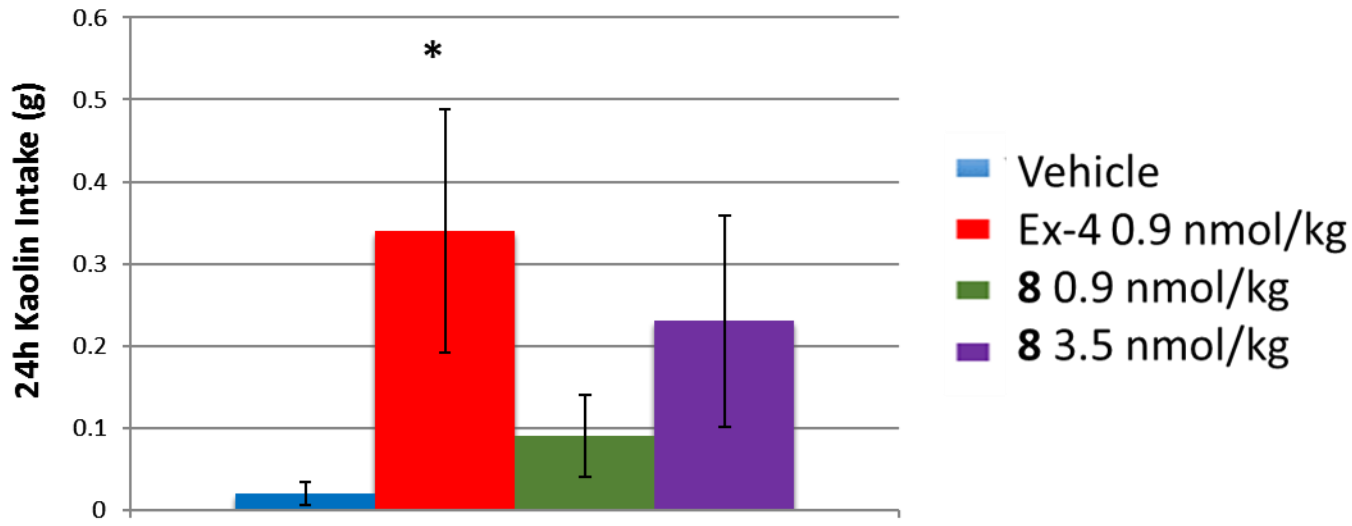


Figure 16. 24 h kaolin intake after Ex-4 at 0.9 nmol/kg and **8** at 0.9 nmol/kg and 3.5 nmol/kg administration (n=6).

2.5 Outcomes and conclusions

The in vitro assays show that **8** and the complex IF-**8** maintain picomolar agonism at the GLP-1R and are both within one log order of the Ex-4 EC_{50}, whether following cAMP directly or indirectly through PKA levels. The modifications to Ex-4 do not significantly change the agonism at the receptor, and the conjugation still allows for binding to the B_{12} binding proteins. In vivo results reveal that the changes to the hydrophilicity have changed the pharmacodynamics of Ex-4. The conjugation to B_{12} reverses the hyperglycemia seen in rats and works to lower the glucose levels. The most significant finding of this chapter is that B_{12} conjugation to Ex-4 shows a smaller decrease of food intake and reduction of nausea. This suggests a blocking of central GLP-1R activation, presumably in the brain, while allowing peripheral GLP-1R activation to produce glucoregulation responses from the pancreas to continue. This work has considerable potential to be a new form of the Ex-4 pharmaceutical without the side-effect of chronic nausea. The goal of this chapter, to remove side-effects of Ex-4 without loss of glucoregulation, was achieved in a rat model.

2.6 Reference

1. Mosenzon, O.; Pollack, R.; Raz, I., Treatment of Type 2 Diabetes: From “Guidelines” to “Position Statements” and Back. *Recommendations of the Israel National Diabetes Council* **2016,** *39* (Supplement 2), S146-S153.

2. Meier, J. J., GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. *Nat Rev Endocrinol* **2012,** *8* (12), 728-742.

3. Kanoski, S. E.; Rupprecht, L. E.; Fortin, S. M.; De Jonghe, B. C.; Hayes, M. R., The role of nausea in food intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide. *Neuropharmacology* **2012,** *62* (5), 1916-1927.

4. Baraboi, E.-D.; St-Pierre, D. H.; Shooner, J.; Timofeeva, E.; Richard, D., Brain activation following peripheral administration of the GLP-1 receptor agonist exendin-4. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **2011,** *301* (4), R1011-R1024.

5. Gejl, M.; Rungby, J.; Brock, B.; Gjedde, A., At the Centennial of Michaelis and Menten, Competing Michaelis–Menten Steps Explain Effect of GLP-1 on Blood–Brain Transfer and Metabolism of Glucose. *Basic & Clinical Pharmacology & Toxicology* **2014,** *115* (2), 162-171.

6. Sisley, S.; Gutierrez-Aguilar, R.; Scott, M.; x; Alessio, D. A.; Sandoval, D. A.; Seeley, R. J., Neuronal GLP1R mediates liraglutide's anorectic but not glucose-lowering effect. *The Journal of Clinical Investigation* **2014,** *124* (6), 2456-2463.

7. Kastin, A. J.; Akerstrom, V., Entry of exendin-4 into brain is rapid but may be limited at high doses. *Int J Obes Relat Metab Disord* **2003,** *27* (3), 313-318.

8. Kastin, A.; Akerstrom, V., Entry of exendin-4 into brain is rapid but may be limited at high doses. *International journal of obesity* **2003,** *27* (3), 313-318.

9. Lai, S.-C.; Nakayama, Y.; Sequeira, J. M.; Wlodarczyk, B. J.; Cabrera, R. M.; Finnell, R. H.; Bottiglieri, T.; Quadros, E. V., The transcobalamin receptor knockout mouse: a model for vitamin B12 deficiency in the central nervous system. *The FASEB Journal* **2013,** *27* (6), 2468-2475.

10. Lazar, G.; Carmel, R., Cobalamin binding and uptake in vitro in the human central nervous system. *The Journal of laboratory and clinical medicine* **1981,** *97* (1), 123-133.

11. Nijst, T.; Wevers, R.; Schoonderwaldt, H.; Hommes, O.; De Haan, A., Vitamin B12 and folate concentrations in serum and cerebrospinal fluid of neurological patients with special reference to multiple sclerosis and dementia. *Journal of Neurology, Neurosurgery & Psychiatry* **1990,** *53* (11), 951-954.

12. Doyle, R.; Clardy-James, S.; Bernstein, J.; Kerwood, D., Site-Selective Oxidation of Vitamin B12 Using 2-Iodoxybenzoic Acid. *Synlett* **2012,** *23* (16), 2363-2366.

13. Henry, K. E.; Elfers, C. T.; Burke, R. M.; Chepurny, O. G.; Holz, G. G.; Blevins, J. E.; Roth, C. L.; Doyle, R. P., Vitamin B12 Conjugation of Peptide-YY3–36 Decreases Food Intake Compared to Native Peptide-YY3–36 Upon Subcutaneous Administration in Male Rats. *Endocrinology* **2015,** *0* (0), en.2014-1825.

14. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angewandte Chemie International Edition* **2001,** *40* (11), 2004-2021.

15. Kolb, H. C.; Sharpless, K. B., The growing impact of click chemistry on drug discovery. *Drug Discovery Today* **2003,** *8* (24), 1128-1137.

16. Huisgen, R., 1, 3-Dipolar cycloadditions-introduction, survey, mechanism. 1, 3-Dipolar cycloaddition chemistry (Padwa, A., Ed.) pp 1-176. Wiley, New York: 1984.

17. Dzhura, I.; Chepurny, O. G.; Leech, C. A.; Roe, M. W.; Dzhura, E.; Xu, X.; Lu, Y.; Schwede, F.; Genieser, H.-G.; Smrcka, A. V.; Holz, G. G., Phospholipase C-ε links Epac2 activation to the potentiation of glucose-stimulated insulin secretion from mouse islets of Langerhans. *Islets* **2011,** *3* (3), 121-128.

18. Thorens, B.; Porret, A.; Bühler, L.; Deng, S.-P.; Morel, P.; Widmann, C., Cloning and Functional Expression of the Human Islet GLP-1 Receptor: Demonstration That Exendin-4 Is an Agonist and Exendin-(9–39) an Antagonist of the Receptor. *Diabetes* **1993,** *42* (11), 1678-1682.

19. Stupperich, E.; NexØ, E., Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin B12 binding proteins intrinsic factor, transcobalamin and haptocorrin. *European Journal of Biochemistry* **1991,** *199* (2), 299-303.

20. Bartoli, E.; Fra, G. P.; Carnevale Schianca, G. P., The oral glucose tolerance test (OGTT) revisited. *European journal of internal medicine* **2011,** *22* (1), 8-12.

21. Siu, A. L., Screening for Abnormal Blood Glucose and Type 2 Diabetes Mellitus: U.S. Preventive Services Task Force Recommendation StatementScreening for Abnormal Blood Glucose and Type 2 Diabetes Mellitus. *Annals of Internal Medicine* **2015,** *163* (11), 861-868.

22. O'Sullivan, J. B.; Mahan, C., Glucose tolerance test. In *Variability in pregnant and non-pregnant women*, 1966; Vol. 19, pp 345-351.

23. Andrikopoulos, S.; Blair, A. R.; Deluca, N.; Fam, B. C.; Proietto, J., Evaluating the glucose tolerance test in mice. *American Journal of Physiology-Endocrinology and Metabolism* **2008,** *295* (6), E1323-E1332.

24. Pérez-Tilve, D.; González-Matías, L.; Aulinger, B. A.; Alvarez-Crespo, M.; Gil-Lozano, M.; Alvarez, E.; Andrade-Olivie, A. M.; Tschöp, M. H.; D'Alessio, D. A.; Mallo, F., Exendin-4 increases blood glucose levels acutely in rats by activation of the sympathetic nervous system. *American Journal of Physiology-Endocrinology and Metabolism* **2010,** *298* (5), E1088-E1096.

25. Pannacciulli, N.; Le, D. S. N. T.; Salbe, A. D.; Chen, K.; Reiman, E. M.; Tataranni, P. A.; Krakoff, J., Postprandial glucagon-like peptide-1 (GLP-1) response is positively associated with changes in neuronal activity of brain areas implicated in satiety and food intake regulation in humans. *NeuroImage* **2007,** *35* (2), 511-517.

26. Field, B. C. T.; Chaudhri, O. B.; Bloom, S. R., Bowels control brain: gut hormones and obesity. *Nat Rev Endocrinol* **2010,** *6* (8), 444-453.

27. Takeda, N.; Hasegawa, S.; Morita, M.; Matsunaga, T., Pica in rats is analogous to emesis: An animal model in emesis research. *Pharmacology Biochemistry and Behavior* **1993,** *45* (4), 817-821.

28. Mitchell, D.; Wells, C.; Hoch, N.; Lind, K.; Woods, S. C.; Mitchell, L. K., Poison induced pica in rats. *Physiology and Behavior* **1976,** *17* (4), 691-697.

29. Garcia, J.; Kimeldorf, D. J.; Koelling, R. A., Conditioned Aversion to Saccharin Resulting from Exposure to Gamma Radiation. *Science* **1955,** *122* (3160), 157-158.

Chapter 3: IF protection of 8 from systemic and pancreatic proteases

Work in this chapter resulted in the publication of: Bonaccorso, R. L.; Chepurny, O. G.; Becker-Pauly, C.; Holz, G. G.; Doyle, R. P., Enhanced Peptide Stability Against Protease Digestion Induced by Intrinsic Factor Binding of a Vitamin B12 Conjugate of Exendin-4. *Molecular Pharmaceutics* 2015, 12 (9), 3502-3506.

3.1 Introduction

The future of pharmaceutics and medicinal chemistry is in peptide therapeutics. Peptides in general offer several advantages over small molecule drugs, which have historically dominated pharmaceutical research and therapeutics, including fewer side effects, greater selectivity, and higher potency.[1] Because of the high selectivity possible with peptides, new therapeutics can be used to target difficult to treat diseases.[2] Peptide drug research is still relatively new, but it is a quickly growing field of pharmaceutical research.[3]

Despite the many benefits of peptide therapeutics, there are several major hurdles facing peptide therapeutics, including rapid degradation through proteolysis, short half-lives and rapid clearance, and low oral bioavailability.[4] One of the many benefits of peptides as therapeutics is increased potency. Unfortunately, without an increase in half-life these peptides offer limited therapeutic value even with their higher potency. One such example of this is GLP-1, which, despite its potency, offers no therapeutic value due to its short half-life.[5] In vivo half-life has been increased through use of albumin and pegylation.[6,7] To increase the pharmaceutical potential of peptide research an effective and easily employable method of increasing oral bioavailability and protease resistance is essential.[8]

Extensive research into increasing oral bioavailability and peptide stability has led to promising results and several drugs using these new methodologies are in clinical trials.[9] There is still no “go to” technique to provide oral delivery and protease resistance.[10] Many of these techniques alter the potency and inactivate the drug. This underlies the reason to have multiple methodologies and techniques to use for peptide delivery and proteolysis resistance.

Most approved peptide medications are taken through injection.[11] The low oral bioavailability of these peptides is due to their potential for degradation by enzymes and proteases.[12] Ex-4, although resistant to degradation in the blood and currently available as a therapeutic, is an example of an injectable peptide prone to degradation by pancreatic proteases.[13] In attempts to prevent degradation, Ex-4 can be used as a probe to detect proteolysis by following GLP-1R agonism after digestion. The level of maintained agonism at the receptor should correlate inversely with the amount of peptide digested.

In chapter 2 I demonstrated that IF binding of B_{12}, upon conjugation to Ex-4, was maintained. It was also demonstrated that functional agonism of GLP-1R was maintained with low picomolar agonism. The question targeted herein is whether IF binding of **8** to a dietary uptake protein can offer protection to the conjugated peptide as well as the B_{12} molecule.[14] B_{12} is a particularly attractive candidate for peptide conjugation since it possesses no inherent negative side effect.[15] The **hypothesis** is that upon complexation IF should shield **8** from proteases thereby protecting it from degradation. IF's natural resistance to proteolysis and encapsulation of B_{12} make it an ideal shield for degradation of conjugates as well.[16,17] To test this hypothesis **8** and IF-**8** need to be incubated in the presence of proteases and then tested for agonism at the GLP-1R. Stability of Ex-4, **8** and IF-**8** against the abundant intestinal endopeptidases, which represent both gastric and systemic proteases (see Table 1), trypsin, chymotrypsin, and meprin β were compared by measuring relative function at the GLP-1R after digestion.[18,19,20] This work will test the value of IF in protecting B_{12} from proteases. This work will show the potential of IF and B_{12} conjugation and possibly provide evidence for oral delivery of therapeutics through the B_{12} dietary pathway.

Table 1. Proteases and where they cut

	Trypsin	**Chymotrypsin**	**Meprin β**
Designation	Basic	Aromatic	Acidic
Location	Gastric	Gastric	Systemic
Digestion Locations	Lysine and Arginine	Tyrosine, Tryptophan and Phenylalanine	Aspartic acid, Glutamic acid

3.2 In vitro testing of IF protection of 8

Ex-4, **8** and IF-**8** were analyzed for stability against proteases by measuring remaining function at the receptor compared to undigested controls. Ex-4, **8** and IF-**8** were tested for function at [100 nM], a concentration at which each had comparable percent change in FRET ratio (see Table 1). After establishing a reference point for 100% function each sample could be tested for agonism relative to 100% function after proteolysis.

Table 2. % Change in FRET at 100 nM for Ex-4, **8** and IF-**8**.

Compound	**% Change in FRET at 100 nM**
Ex-4	-12± 0.01
8	-13± 0.02
IF-**8**	-12± 0.01

Digestion was conducted in a standard extracellular solution containing either trypsin at 11, 22, or 50 μg/mL, chymotrypsin at 1.25, 3, or 6.25 μg/mL or meprin β at 2 or 10 μg/mL see Figure 1 and Figure 2. Three different concentrations of the pancreatic proteases were used to track the relative differences between **8** and IF-**8** (see Figure 1). At the lowest concentration of trypsin (11 μg/mL) and chymotrypsin (1.25 μg/mL) there is up to 50% greater function for IF-**8** relative to **8** alone with the highest concentration of trypsin (50 μg/mL) and chymotrypsin (6.25 μg/mL) assayed showing complete lack of function for all systems. The digestion was monitored by measuring agonism of the drugs at the GLP-1R, initially over the

course of 3 h, although it was quickly noted that there was no change after 1.5 h indicating that the digestion had stopped by this time point (data not shown). Subsequent triplicate runs were then performed on digestions of 1.5 h.

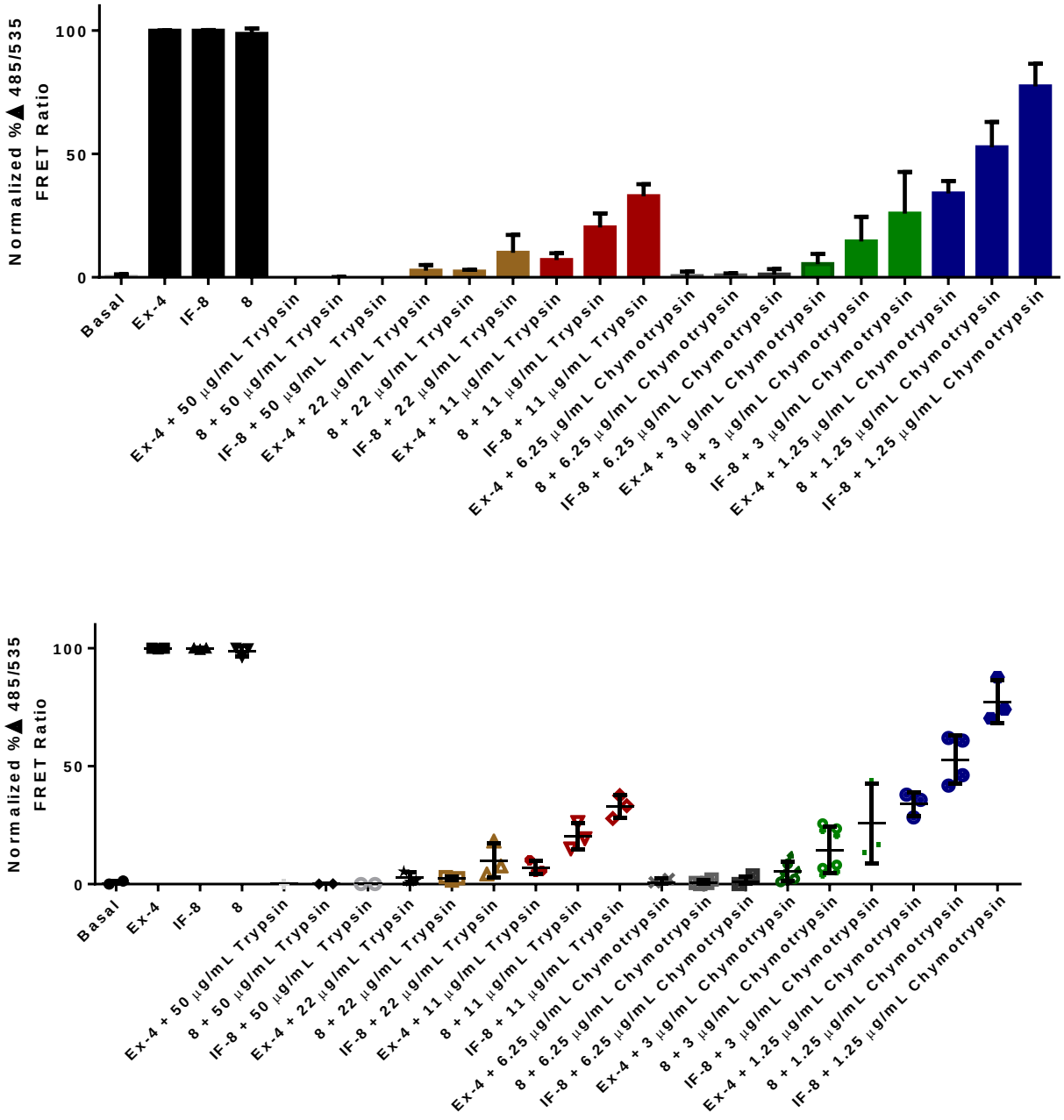


Figure 1. Top: column graph and Bottom: scatter plot of 1.5 h trypsin and chymotrypsin digestion of 100 nM Ex-4, **8** and IF-**8** with 50, 22 or 11 µg/mL of trypsin or 1.25, 3, or 6.25 µg/mL of chymotrypsin using

AKAR3 to measure function. The data shows the maximum expression normalized to 100% of the conjugates done in triplicate (mean ± SEM). Basal control contained trypsin at 50 µg/mL of trypsin. (N.C. = no change).

Meprin β digestion revealed a 3.3 fold increase in function with B_{12} conjugation and a 4.5 fold increase in function when pre-bound to IF (see Figure 5). No function was seen for Ex-4 at concentrations greater than 3 µg/mL. The protection provided from B_{12} conjugation and subsequent binding to IF show that key residues are being protected. Results of the AKAR3 assays show maintenance of function where otherwise none was observed or improvement of function when 4 is first bound to IF. B_{12} conjugation itself offered some protection at the low trypsin concentrations tested.

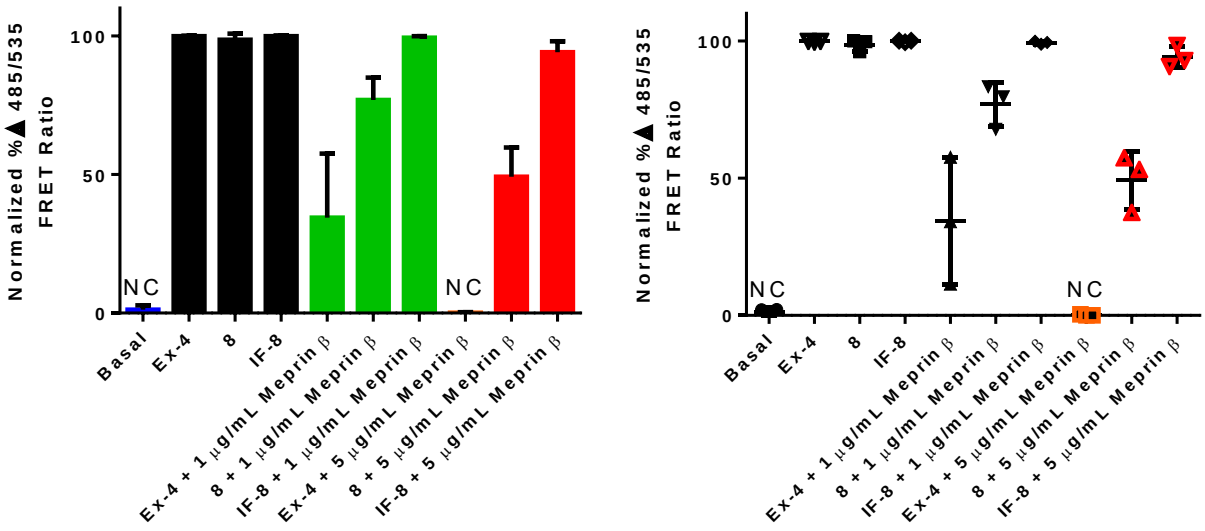


Figure 2. Left: column graph and Right: scatter plot of 30 min meprin β digestion of 100 nM Ex-4, **8** and IF-**8** with 2 and 10 µg/mL of meprin β. The data shows the maximum expression normalized to 100% of the conjugates done in triplicate (mean ± SEM). Basal control contained 2 µg/mL of meprin β. Recombinant human meprin β was produced in insect cells and purified and activated as described previously.

3.3 Outcomes and conclusions

The conservation or improved relative function demonstrated herein for Ex-4 when conjugated to B_{12} and more significantly, when bound by IF is an important first-step in addressing the use, and putative role, of IF in delivering and protecting an orally administered peptide. Protection against pancreatic protease-catalyzed hydrolytic digestion of **8** was maximal at an optimal trypsin concentration of 22 μg /mL of trypsin and 3 μg/mL of chymotrypsin when **8** was pre-bound to IF, providing a 4-fold and 7.5-fold positive increase in function respectively as measured by GLP-1R agonism (utilizing a new AKAR3 screening assay). The digestion with metalloendoprotease meprin β showed the most significant protection when comparing Ex-4 and IF-**8**. No reduction in function was seen at the highest concentration of meprin β while 1 showed no function at concentrations greater than 2 μg/mL of meprin β. B_{12} provided some protection against trypsin relative to the native peptide. The effect is seen at 11 μg/mL of trypsin with a relative increase of 3-fold and at 1.25 and 3 μg/mL of chymotrypsin with a relative increase of 3 and 1.6-fold. The fact that the IF bound form IF-**8** still maintained significant function at the GLP-1R is also highly significant since many routes to protect against intestinal degradation involve encapsulation, which prevents possible luminal function or absorption when in place. The use of IF to improve the protease stability of a peptide offers significant scope for exploitation. Even a small improvement in oral function may be sufficient to achieve the targeted effect. Combining this approach with a highly potent peptide with known gut receptors that can produce a vagal afferent response (such as, but not limited to, GLP-1/Ex-4 or PYY(3-36)) for example, may allow for a positive clinical outcome to be achieved orally, without need even for systemic delivery. Finally, as demonstrated by the stability against meprin β, there is no suggestion that this approach is limited to use against gastric proteases, but could also be expanded into serum (through intravenous injection of IF bound peptide conjugates for instance), facilitating greatly improved pharmacokinetics, making this a possible platform technology for peptide drug delivery. The

goal of this chapter, to test that IF binding of **8** confers protection against gastric proteolysis as a road map to oral peptide delivery.

3.4 References

1. Fosgerau, K.; Hoffmann, T., Peptide therapeutics: current status and future directions. *Drug Discovery Today* **2015,** *20* (1), 122-128.
2. Tsomaia, N., Peptide therapeutics: Targeting the undruggable space. *European Journal of Medicinal Chemistry* **2015,** *94*, 459-470.
3. Kent, S. B. H., The critical role of peptide chemistry in the life sciences. *Journal of Peptide Science* **2015,** *21* (3), 136-138.
4. Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatisky, M., Synthetic therapeutic peptides: science and market. *Drug Discov Today* **2010,** *15* (1-2), 40-56.
5. Hausenloy, D. J.; Yellon, D. M., GLP-1 Therapy: Beyond Glucose Control. *Circulation: Heart Failure* **2008,** *1* (3), 147-149.
6. Sleep, D.; Cameron, J.; Evans, L. R., Albumin as a versatile platform for drug half-life extension. *Biochimica et Biophysica Acta (BBA) - General Subjects* **2013,** *1830* (12), 5526-5534.
7. Werle, M.; Bernkop-Schnürch, A., Strategies to improve plasma half life time of peptide and protein drugs. *Amino Acids* **2006,** *30* (4), 351-367.
8. Veber, D. F.; Johnson, S. R.; Cheng, H.-Y.; Smith, B. R.; Ward, K. W.; Kopple, K. D., Molecular Properties That Influence the Oral Bioavailability of Drug Candidates. *Journal of medicinal chemistry* **2002,** *45* (12), 2615-2623.
9. Aguirre, T. A. S.; Teijeiro-Osorio, D.; Rosa, M.; Coulter, I. S.; Alonso, M. J.; Brayden, D. J., Current status of selected oral peptide technologies in advanced preclinical development and in clinical trials. *Advanced Drug Delivery Reviews*.
10. Bak, A.; Leung, D.; Barrett, S. E.; Forster, S.; Minnihan, E. C.; Leithead, A. W.; Cunningham, J.; Toussaint, N.; Crocker, L. S., Physicochemical and Formulation Developability Assessment for Therapeutic Peptide Delivery—A Primer. *The AAPS Journal* **2015,** *17* (1), 144-155.
11. Renukuntla, J.; Vadlapudi, A. D.; Patel, A.; Boddu, S. H. S.; Mitra, A. K., Approaches for enhancing oral bioavailability of peptides and proteins. *International Journal of Pharmaceutics* **2013,** *447* (1–2), 75-93.
12. Hamman, J. H.; Enslin, G. M.; Kotzé, A. F., Oral Delivery of Peptide Drugs. *BioDrugs* **2005,** *19* (3), 165-177.
13. Mentlein, R.; Gallwitz, B.; Schmidt, W. E., Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7–36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *European Journal of Biochemistry* **1993,** *214* (3), 829-835.
14. Fedosov, S. N.; Berglund, L.; Fedosova, N. U.; Nexø, E.; Petersen, T. E., Comparative Analysis of Cobalamin Binding Kinetics and Ligand Protection for Intrinsic Factor, Transcobalamin, and Haptocorrin. *Journal of Biological Chemistry* **2002,** *277* (12), 9989-9996.
15. Nyholm, E.; Turpin, P.; Swain, D.; Cunningham, B.; Daly, S.; Nightingale, P.; Fegan, C., Oral vitamin B12 can change our practice. *Postgraduate Medical Journal* **2003,** *79* (930), 218-219.
16. Seetharam, B.; Alpers, D.; Allen, R., Isolation and characterization of the ileal receptor for intrinsic factor-cobalamin. *J Biol Chem* **1981,** *256* (8), 3785-3790.
17. Andersen, K. J.; Von Der Lippe, G., The Effect of Proteolytic Enzymes on the Vitamin B12-binding Proteins of Human Gastric Juice and Saliva. *Scandinavian journal of gastroenterology* **1979,** *14* (7), 833-838.

18. Broder, C.; Becker-Pauly, C., The metalloproteases meprin α and meprin β: unique enzymes in inflammation, neurodegeneration, cancer and fibrosis. *Biochemical Journal* **2013,** *450* (Pt 2), 253-264.
19. Appel, W., Chymotrypsin: Molecular and catalytic properties. *Clinical Biochemistry* **1986,** *19* (6), 317-322.
20. Hustoft, H. K.; Malerod, H.; Wilson, S. R.; Reubsaet, L.; Lundanes, E.; Greibrokk, T., A critical review of trypsin digestion for LC-MS based proteomics. *Integrative Proteomics* **2012,** *1*, 73-82.

Chapter 4: Rationally designed GLP-1R/Y2R agonist peptide

The work in this chapter was done in collaboration with Professor George G. Holz and Dr. Christian L. Roth. The work reported in this chapter will be submitted to *PNAS* with the title "Diabetes and weight loss therapy through hybrid peptides" and coauthors: Ron L. Bonaccorso, Clinton L. Elfers, George G. Holz, Christian L. Roth and Robert P. Doyle

The work from this chapter resulted in a patent pending: SU100846_156P471

4.1 Introduction

Weight loss research has been given a lot of attention in recent years due to the growing epidemic of obesity.[1] Despite all of the research and therapeutic options, effective weight loss treatments have been difficult to find.[2] There are only five FDA approved drugs marketed for treatment of obesity but the effectiveness of these drugs are variable and not consistent from patient to patient.[3] Several diabetes medications have been shown to cause weight loss and recently liraglutide has even been approved as a weight loss medication.[4] Most of these drugs show limited effectiveness, and many potential drugs either lose effectiveness over time or result in significant weight gain after treatment has ended.[5] The body is able to invoke a counter-regulatory response and ignore the signals suppressing appetite deeming the drug ineffective.[6]

Studies have shown that combination therapy can be effective at treating obesity.[7] Drug cocktails however are difficult and expensive to test since there are multiple PK and PD pathways to test and side effects to consider. A single peptide that targets multiple receptors is an ideal candidate to treat obesity.[8] Combination treatments consisting of GLP-1R agonists and NPY2R agonists have shown to be particularly effective at treating both diabetes and obesity.[8] Coagonism of GLP-1R and either the glucagon receptor (GCGR) or GIPR have shown to have enhanced glucose and weight loss control as well.[9,10] Current co-

agonists have shown to be effective in vitro but have limited efficacy in vivo. T2DM and obesity management through combination therapies have potential to be effective and are expected to be the future of weight control therapeutics.[11]

4.2 Design of hybrid proteins

To overcome the compensatory effects of PYY(3-36), a hybrid peptide, made from fragments of Ex-4 and PYY(3-36), should be able to target both GLP-1R and NPY2R without the PD effects of two different drugs. A series of peptides were designed with the goal of combining fragments of Ex-4, which were vital for GLP-1R agonism, and fragments of PYY(3-36), which are necessary for maintaining agonism to the NPY2R.[12] The similarity between glucagon receptor superfamily peptides opens the possibility of receptor agonism at GIPR and GCGR.[13,14] An initial series of three sequences were designed with the first two peptides composed primarily of Ex-4 sequence and the third as a modified version of PYY(3-36). Table 1 shows the comparison of each peptide in relation to Ex-4 and PYY. An initial series of 3 peptides, RLB001 (**11**), RLB002 (**12**) and RLB003 (**13**), was designed to screen for dual agonism. After the results of these three peptides an additional three, RLB004 (**14**), RLB005 (**15**) and RLB006 (**16**), were designed. The last three peptides were designed to try and improve upon the initial series of **11**, **12** and **13** to produce an effective dual or even triagonist.[15]

Table 1. Hybrid peptide design and comparison. Sequences corresponding to **11**-**16** were designed with elements of PYY(3-36) and Ex-4. Amino acid sequence taken from PYY(3-36) is shown in blue and amino acid sequence taken from Ex-4 is shown in black.

Peptide	Sequence
PYY(3-36)	IKPEAPREDASPEELNRYYASLRHYLNLVTRQRY-NH2
Ex-4	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH2
(11) RLB001	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQRY-NH2
(**12**) RLB002	HGEGTFTSDLSKQMEEEAVRLFIEWLRHYLNLVTRQRY-NH2
(**13**) RLB003	IKPEAPREDASPEENQAYKEFIAYLNLVTRQRY-NH2
(**14**) RLB004	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSRHYLNLVTRQRY-NH2
(**15**) RLB005	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQ-NH2
(**16**) RLB006	HGEGTFTSDLSK(azido)QMEEEAVRLFIEWLKNGGPSSTRQRY-NH2

4.3 In vitro screening and testing

The RLB series peptides were tested for function at receptors corresponding to the glucagon super family of receptors and NPY receptors. These receptors were transfected into either HEK293 cells or CHO cells. HEK 293 cells stably transfected with either GLP-1R or GIPR followed by an infection of adenovirus incorporating AKAR3 to indirectly follow cAMP production. CHO cells were transfected with NPY2R and a promiscuous g protein using FURA2 to follow calcium release.

4.3.1 Screening the RLB series for GLP-1R agonism and dose response

Function was first established at the GLP-1R by screening the initial conjugates in the HEK-GLP-1R cells. These cells were treated with adenovirus containing AKAR3 and a agonism was determined by a decrease in FRET ratio. The initial screen of compounds is found in Figure 1 and shows that **11**, **12** and **14** have agonism at the GLP-1R. **13** showed no function at the GLP-1R at 300 nM making ineffective as a GLP-1R and NPY2R coagonist.

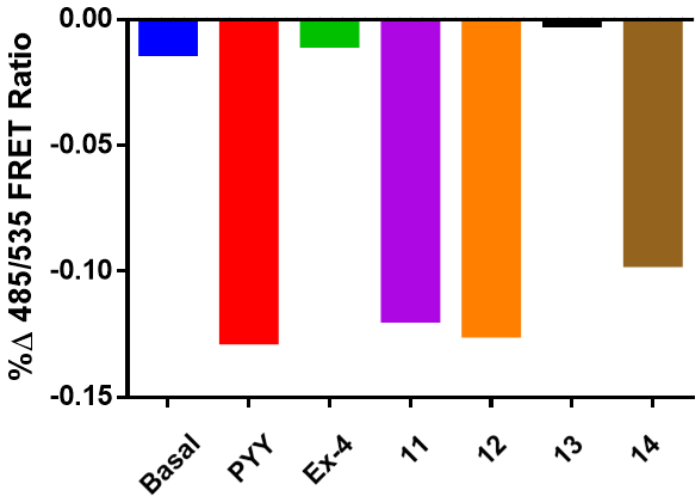


Figure 1. A screen of RLB series peptides at the GLP-1R at 300 nM.

The initial screen showed that modifications to Ex-4 don't completely hinder function at the GLP-1R. The peptides showing function at the GLP-1R were then analyzed further to establish an EC_{50}. Figure 2 shows that **11** and **14** are the most effective with EC_{50} values of 50 ± 3 pM and 128 ± 30 pM, respectively and followed by **12** at 253 ± 50 pM.

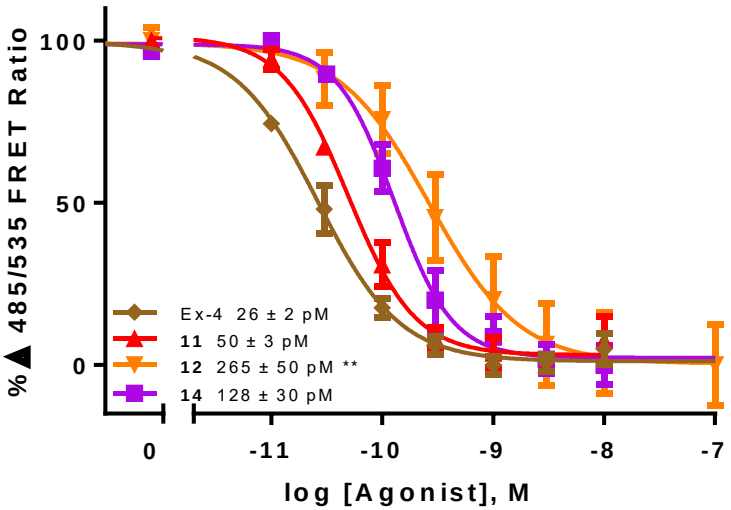


Figure 2. Dose response comparison of **11**, **12** and **14** to Ex-4. **12** has a p-value of 0.0032 compared to Ex-4 and **11**.

11, **12** and **14** all retained picomolar agonism at GLP-1R warranting further analysis at NPY2R and other glucagon superfamily receptors. **13** was discarded as a potential dual agonist due to its loss of function.

4.3.2 Screening the RLB series for NPY2R agonism and dose response

Following a screen at the GLP-1R, a screen for agonism at NPY2R was conducted. Figure 4 shows full function was only achieved with **12** and **14**. Positive results show that a successful in vitro dual agonist of GLP-1R and NPY2R was achieved.

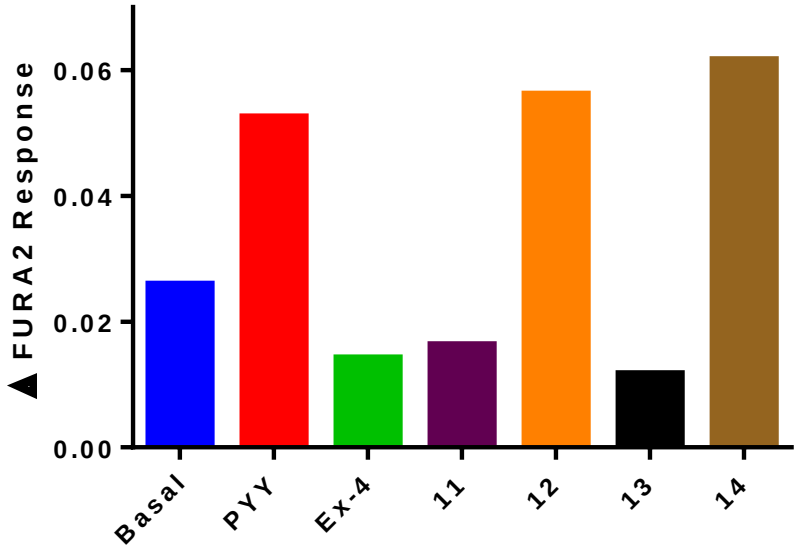


Figure 3. NPY2R screen with RLB series of peptides at [300 nM].

12 and **14** were followed up with a full dose response curve to establish an EC_{50} for each peptide (see Figure 4). PYY(3-36) was used as the positive control to show that each peptide shows comparable agonism at the NPY2R through in vitro trials. **14** had an EC_{50} of 107 ± 9 nM and **12** had an EC_{50} of 59 nM.

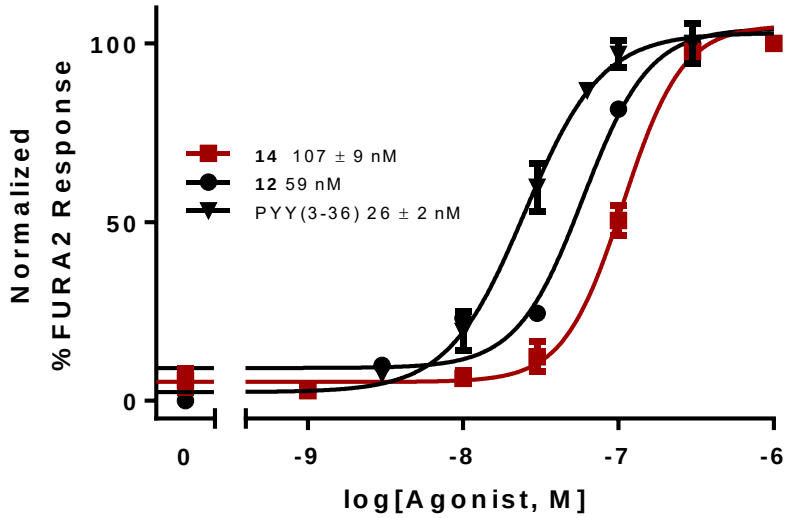


Figure 4. NPY2R EC_{50}s of **12**, 59 nM, and **14**, 107 nM.

4.3.3 Screening the RLB series for GIPR and GCGR agonism and dose response

After establishing agonism at the GLP-1R, the RLB series peptides were tested against similar receptors. Receptors in the glucagon receptor superfamily were tested based on the similarities of native peptide agonists. Shown in Figure 5 are the results of the GIPR screen, where only **11** and **15** showed function at the GIPR and Ex-4, PYY, **12** and **14** showed no function under 1 µM.

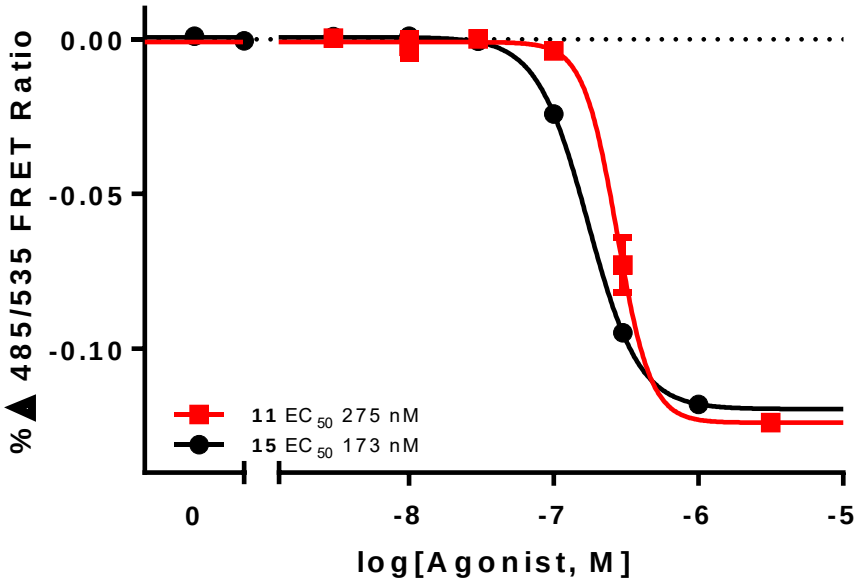


Figure 5. GIPR dose response and EC_{50} values of **11** (275 nM) and **15** (173 nM). Note: Ex-4, **12**, **14** and PYY were all tested with no agonism up to 1 µM.

Peptides **11** and **14** were screened for GCGR agonism compared to Ex-4. Each of these peptides, including Ex-4, showed slight agonism at the receptor at high concentrations (>300 nM). Only peptides **11**, **12** and **14** were confirmed to have function at more than one receptor. **11** is an effective in vitro dual agonist of GLP-1R and GIPR and **12** and **14** are effective agonists of GLP-1R and NPY2R.

4.4 In vivo testing of 11 compared to Ex-4 and PYY(3-36)

After the initial screening of the RLB series peptides an in vivo study was planned to compare the dual agonists **11** and **14** to Ex-4 and PYY(3-36). Since **12** was significantly less potent at the GLP-1R it was not selected for in vivo testing. A one-day food intake screen of **11** and **14** showed that **11** had a reduction in food intake but **14** actually resulted in the opposite effect and caused an increased food intake from the rats (see Figure 6). Given the initial results only **11** was pursued.

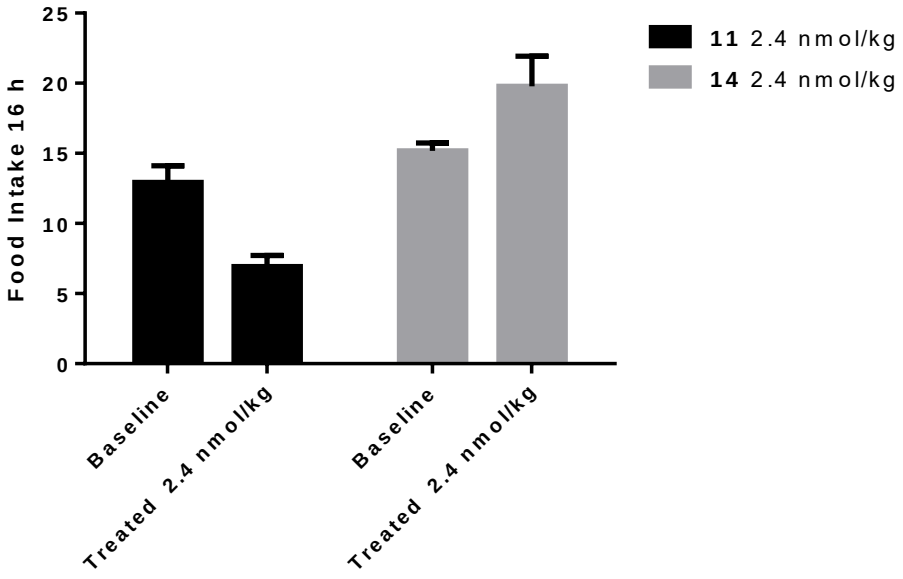


Figure 6. Initial screen of food intake for compounds **11** and **14** at 2.4 nmol/kg over 16 h.

11 was isolated as the lead candidate for both blood glucose control and weight loss. A dose response study in was then completed in rats to determine the optimal concentration of **11.** A two day food intake study was conducted to verify that **11** behaved uniquely when compared to Ex-4 and co-administration of Ex-4 and PYY(3-36). Figure 7 shows the results of a two day food intake study comparing **11**, Ex-4 and a mixture of Ex-4 and PYY(3-36) all at 2.4 nmol/kg per day. The total reduction of food intake for all three samples is shown in Figure 8. **11** shows a significant improvement over Ex-4 alone and an average lower food intake when compared to Ex-4 and PYY(3-36) co-administration. This establishes that **11** is not Ex-4 but rather a new and different drug.

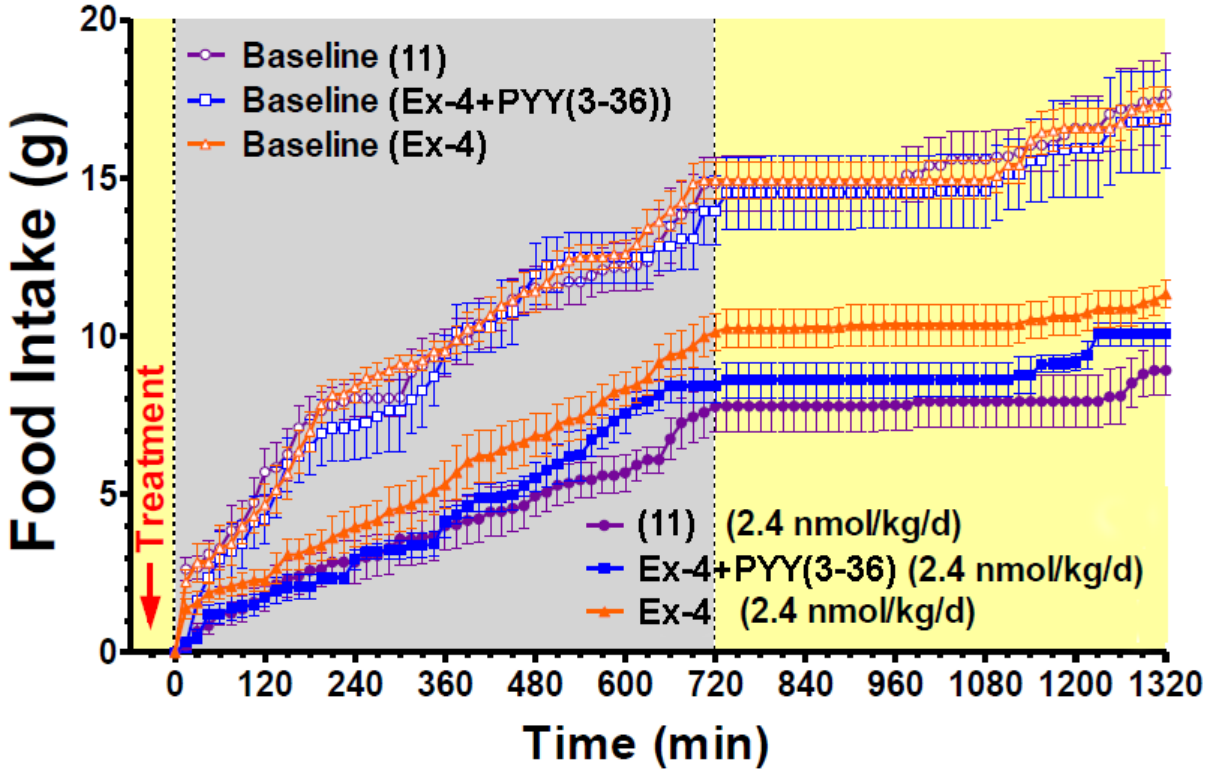


Figure 7. Inhibition of food intake of **11** compared to Ex-4 and Ex-4 and PYY(3-36) combined each at 2.4 nmol/kg/d.

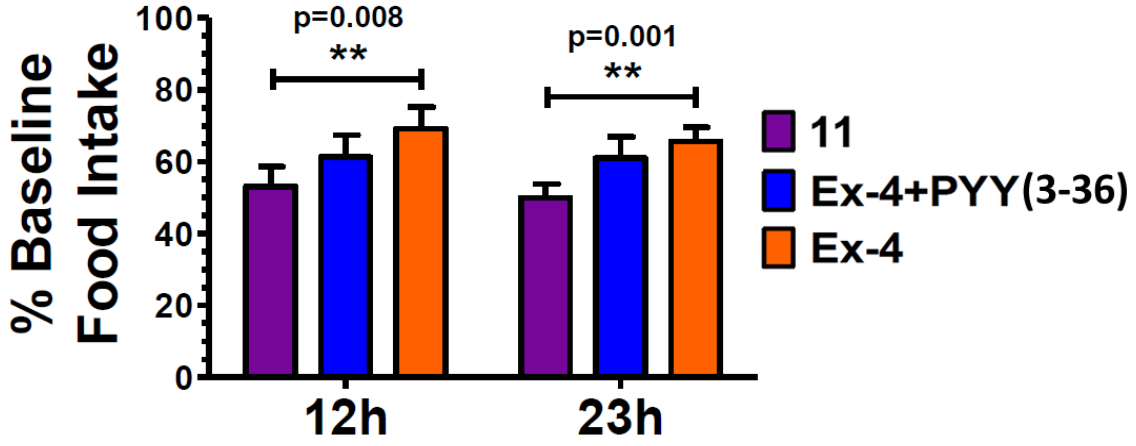


Figure 8. Normalized food intake against baseline levels for **11**, Ex-4 and Ex-4 and PYY(3-36) shown over 12 and 23 h.

Food intake was studied further in diet induced obese (DIO) rats over two days comparing **11**'s treatment at 2.4 nmol/kg to an equimolar mixture of Ex-4 and PYY(3-36) at 2.4 nmol/kg (shown in Figure 9). In each category **11** is out performing Ex-4 and PYY(3-36) combination treatments.

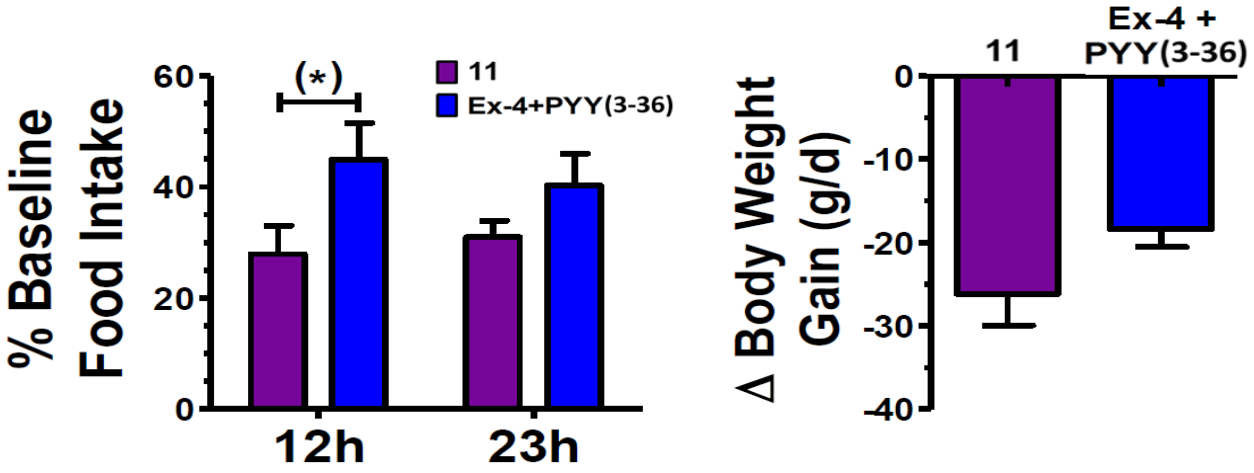


Figure 9. On the left is shown food intake over a two day study with DIO rats administered either 2.4 nmol/kg of **11** or an equimolar mixture of Ex-4 and PYY(3-36). On the right is shown the change in body weight gain of the **11** treatment vs the equimolar Ex-4 and PYY(3-36).

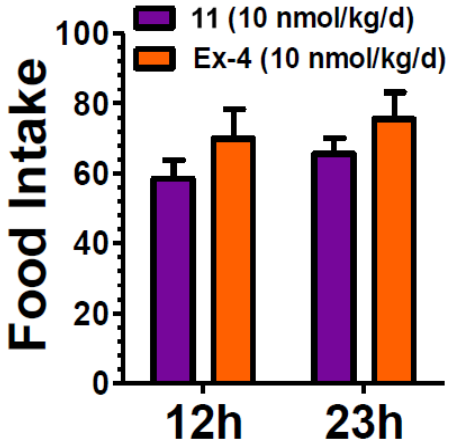


Figure 10. Food intake comparison for **11** and Ex-4 at 10 nmol/kg/d over two days normalized to pretreatment baseline in young 9 week old rats (3 males and 2 females per group).

Direct comparison of **11** and Ex-4 at 10 nmol/kg dosing is seen in Figure 10. **11** is able to provide an average decrease in food intake lower than Ex-4 at both 12 h and 23 h time points.

4.5 Glucoregulatory effects of 11 versus Ex-4

11 was compared directly to Ex-4 to determine efficacy in blood glucose control. An intraperitoneal glucose tolerance test was performed on rats to treated with **11**, Ex-4 and saline (see Figure 11). Both Ex-4 and **11** showed a similar prolonged increase in glucose levels characteristic of Ex-4 in rats. This indicates that the mechanism of action for **11** to control glucose is the same as Ex-4.

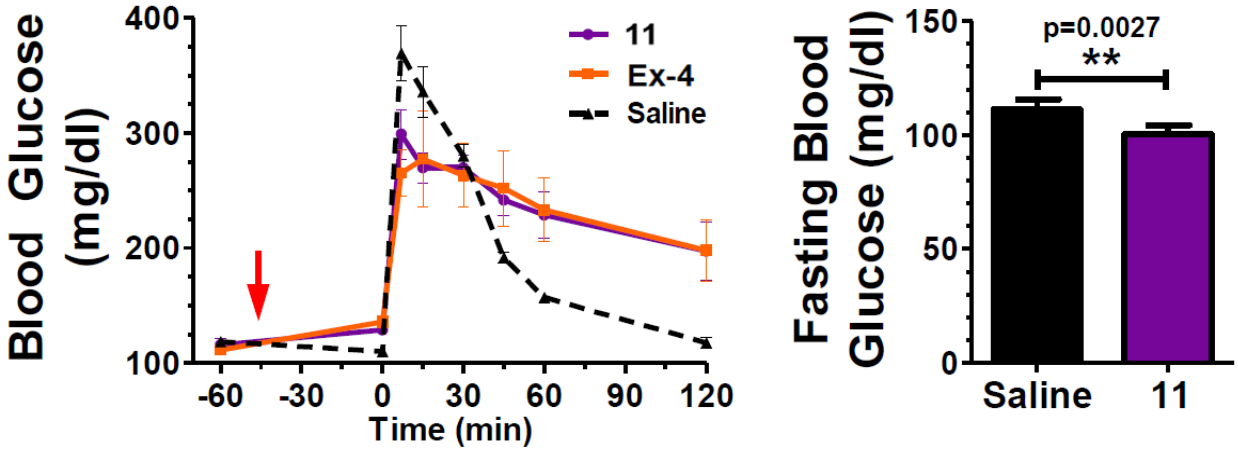


Figure 11. (Left) Blood glucose levels after administration of saline, **11** and Ex-4 following a 10 day treatment (10 nmol/kg/d, 2 males and 2 females per group). (Right) Fasting glucose level reduction after a two day treatment with 10 nmol/kg/d **11** in 31-wk old male rats.

4.6 Outcomes and conclusions

A summary of the results from the in vitro agonism of each receptor is highlighted in table 2. In vitro results were able to confirm dual agonist function with **11** at the GLP-1R and GIPR and dual agonist function with peptides **12** and **14** at the GLP-1R and NPY2R. **11** and **14** were the most potent GLP-1R agonist in vitro and were initially chosen for in vivo trials. No peptide showed any significant function at the NPY1R or the glucagon receptor.

Table 2. Summary of receptor agonism results. (NT= Not tested, ***11** and **14** saw no change in EC50 after 3 months in solution, ****B**$_{12}$-**16** was tested in place of **16**.

Peptide	EC_{50} at Receptors Tested				
	NPY1	NPY2	GLP-1	GIP	Glugagon
PYY(3-36)	>300 nM	16 nM	NT	NT	NT
Ex-4	> 1 μM	>300 nM	26 pM	> 1 μM	>300 nM
*(11)	> 1 μM	>300 nM	50 pM	275 nM	>300 nM
(**12**)	> 1 μM	59 nM	265 pM	> 1 μM	NT
(**13**)	> 1 μM	>300 nM	> 1 μM	> 1 μM	NT
*(**14**)	> 1 μM	107 nM	97 pM	> 1 μM	>300 nM
(**15**)	NT	NT	44 pM	173 nM	NT
(16**)	NT	NT	127 pM	NT	NT

The in vivo results showed that **14** had the reverse effect on appetite and weight loss and caused an increase in both. **11** despite no NPY2R agonism in vitro showed improved efficacy at reducing weight gain and suppressing appetite. **11** even showed improved function over combination therapy of Ex-4 and PYY(3-36) at equimolar dosing at controlling food intake and weight reduction. **11** and Ex-4 showed similar profiles in their glucoregulatory abilities though. **11,** although similar in glucose control, possesses an enhanced ability to control appetite and promote weight loss. There is a need for effective treatment of obesity and T2DM and the initial testing of **11** shows that it there is great potential for it as a lead drug candidate. The goal of this chapter was to create a new lead therapeutic that simultaneously treats diabetes and obesity and this was accomplished.

4.7 References

1. MacLean, P. S.; Wing, R. R.; Davidson, T.; Epstein, L.; Goodpaster, B.; Hall, K. D.; Levin, B. E.; Perri, M. G.; Rolls, B. J.; Rosenbaum, M.; Rothman, A. J.; Ryan, D., NIH working group report: Innovative research to improve maintenance of weight loss. *Obesity* **2015,** *23* (1), 7-15.

2. Domecq, J. P.; Prutsky, G.; Leppin, A.; Sonbol, M. B.; Altayar, O.; Undavalli, C.; Wang, Z.; Elraiyah, T.; Brito, J. P.; Mauck, K. F.; Lababidi, M. H.; Prokop, L. J.; Asi, N.; Wei, J.; Fidahussein, S.; Montori, V. M.; Murad, M. H., Drugs Commonly Associated With Weight Change: A Systematic Review and Meta-analysis. *The Journal of Clinical Endocrinology & Metabolism* **2015,** *100* (2), 363-370.

3. Daneschvar, H. L.; Aronson, M. D.; Smetana, G. W., FDA-Approved Anti-Obesity Drugs in the United States. *The American Journal of Medicine* **2016,** *129* (8), 879.e1-879.e6.

4. Potts, J. E.; Gray, L. J.; Brady, E. M.; Khunti, K.; Davies, M. J.; Bodicoat, D. H., The effect of glucagon-like peptide 1 receptor agonists on weight loss in type 2 diabetes: a systematic review and mixed treatment comparison meta-analysis. *PloS one* **2015,** *10* (6), e0126769.

5. Ross Middleton, K. M.; Patidar, S. M.; Perri, M. G., The impact of extended care on the long-term maintenance of weight loss: a systematic review and meta-analysis. *Obesity Reviews* **2012,** *13* (6), 509-517.

6. Roth, J. D.; Trevaskis, J. L.; Turek, V. F.; Parkes, D. G., "Weighing in" on synergy: Preclinical research on neurohormonal anti-obesity combinations. *Brain Research* **2010,** *1350*, 86-94.

7. Yanovski, S. Z.; Yanovski, J. A., Long-term drug treatment for obesity: A systematic and clinical review. *JAMA* **2014,** *311* (1), 74-86.

8. Sadry, S. A.; Drucker, D. J., Emerging combinatorial hormone therapies for the treatment of obesity and T2DM. *Nature Reviews Endocrinology* **2013,** *9* (7), 425-433.

9. Clemmensen, C.; Chabenne, J.; Finan, B.; Sullivan, L.; Fischer, K.; Küchler, D.; Sehrer, L.; Ograjsek, T.; Hofmann, S. M.; Schriever, S. C., GLP-1/glucagon coagonism restores leptin responsiveness in obese mice chronically maintained on an obesogenic diet. *Diabetes* **2014,** *63* (4), 1422-1427.

10. Skow, M. A.; Bergmann, N. C.; Knop, F. K., Diabetes and obesity treatment based on dual incretin receptor activation:'twincretins'. *Diabetes, Obesity and Metabolism* **2016**.

11. Van Gaal, L.; Scheen, A., Weight Management in Type 2 Diabetes: Current and Emerging Approaches to Treatment. *Diabetes Care* **2015,** *38* (6), 1161-1172.

12. Lerch, M.; Mayrhofer, M.; Zerbe, O., Structural similarities of micelle-bound peptide YY (PYY) and neuropeptide Y (NPY) are related to their affinity profiles at the Y receptors. *Journal of molecular biology* **2004,** *339* (5), 1153-1168.

13. Harmar, A. J., Family-B G-protein-coupled receptors. *Genome Biology* **2001,** *2* (12), 1-10.

14. Seino, Y.; Fukushima, M.; Yabe, D., GIP and GLP‐1, the two incretin hormones: similarities and differences. *Journal of diabetes investigation* **2010,** *1* (1‐2), 8-23.

15. Finan, B.; Yang, B.; Ottaway, N.; Smiley, D. L.; Ma, T.; Clemmensen, C.; Chabenne, J.; Zhang, L.; Habegger, K. M.; Fischer, K.; Campbell, J. E.; Sandoval, D.; Seeley, R. J.; Bleicher, K.; Uhles, S.; Riboulet, W.; Funk, J.; Hertel, C.; Belli, S.; Sebokova, E.; Conde-Knape, K.; Konkar, A.; Drucker, D. J.; Gelfanov, V.; Pfluger, P. T.; Muller, T. D.; Perez-Tilve, D.; DiMarchi, R. D.; Tschop, M. H., A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. *Nat Med* **2015,** *21* (1), 27-36.

Chapter 5: Experimental

5.1 Materials and Methods

The chemicals and solvents were purchased from Sigma-Aldrich, VWR, or Thermo Fisher Scientific and used without further purification. Cyanocobalamin (B_{12}), 2-iodoxybenzoic acid (IBX), 2-hydroxypyridine (HYP), dimethyl sulfoxide (DMSO), propargyl amine, 1-amino-3-butyne, 1-amino-4-pentyne, 1-amino-5-hexyne, 1,1'-carbonyldi(1,2,4-triazole), trimethylamine (TEA), carbodiimide, hydroxybenzotriazole (HOBt), dimethyl formamide (DMF), copper(II) sulfate ($CuSO_4$) sodium ascorbate, and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma Aldrich. Black costar plates with a clear bottom were purchased from Thermo Fisher Scientific. PYY(3-36) K4-azido, PYY(1-36), Ex-4 K12-azido, **11**, **12** and **13** were purchased from C.S. Bio Lab (Cambridge, MA). **14**, **15**, **16** and **14** azido were all purchased from NeoBiolab (Cambridge, MA). H_2O was distilled and deionized using an 18.2 mΩ Barnstead Nano Diamond ultra-purification system. Fetal bovine serum (FBS), PenStrep, Dulbecco's modified eagle media (DMEM), F-12K, Pluronic F-127 and Fura-2 acetoxymethyl ester (Fura-2AM) were purchased from Invitrogen Life Technologies, Carlsbad, CA. Acetonitrile, methanol and tetraflouro acetic acid (TFA) were purchased from VWR. In vitro plate assays were completed using a Molecular Devices FlexStation III running SoftMaxPro software. Xeragenix supplied the human intrinsic factor, which was expressed in *Arabidopsis thaliana*.

RP-HPLC was performed using either an Agilent 1100 system or a Shimadzu Prominence with an Agilent Eclipse C_{18} XBD analytical column (5 µm x 4.6mm x 150 mm). Fast protein liquid chromatography (FPLC) was performed using an AKTAprime plus (GE Healthcare) liquid chromatograph with an automated fraction collector and 5 mL diethyleaminoethyl (DEAE) anion-exchange (AE) column.

MALDI-ToF MS was performed on a Bruker Autoflex III Smartbeam with a laser intensity between 10-20% for samples smaller than 2,000 Da (B_{12} precursor compounds) and between 40-50% for samples larger than 2,000 Da (peptides and bioconjugates). A CHCA matrix was used for all samples and was prepared by

dissolving 10 mg of CHCA to a 50:50 mixture of water and acetonitrile containing 0.1% TFA. Samples were then analyzed by MALDI-ToF-MS using a 50:50 mixture of sample to matrix.

5.2 B_{12}-Ex-4 Synthesis

5.2.1 Synthesis of B_{12}-Carboxylic Acid

1 (1 equivalent), IBX (2.5 equivalents) and HYP (5 equivalents) were dissolved in DMSO at 60 ^{0}C for 3 h. The reaction was then precipitated out using 15:1 diethyl ether and acetone. The crude reaction was then dissolved in H_2O and purified by FPLC using 100% water to bind **2** to the DEAE AE column and 2% NaCl in water to elute pure **2**.

5.2.2 Synthesis of B_{12}-Alkyne Compounds (3-6)

2 was combined with 10 equivalents of EDCl and 20 equivalents of HOBt in dry DMSO under argon and stirred for 20 minutes. To this reaction 10 equivalents of 1-amino-3-butyne was added. This reaction proceeded for 16 h and was then precipitated out with the addition of acetone and ether at a 4:9 ratio. This reaction was separated by HPLC with a flow rate of 1 mL/min and a 1 mL/min gradient of 0-13% acetonitrile over 13 minutes with a retention time of 5 min.

5.2.3 Synthesis of B_{12}-Ex-4 compounds (7-10)

Ex-4 was then conjugated to **2** using copper-catalyzed alkyne-azide cycloaddition.[1] This conjugation was achieved by dissolving Ex-4-K12-azido with either **3**, **4**, **5**, or **6** in a 1:3 ratio, respectively with copper(II) sulfate ($CuSO_4$) and sodium ascorbate in water/DMF (4:1) and stirring for 1 h. The reaction yields were greater than 90% with isolated purity greater than 95% for **6-9** as indicated by HPLC in all cases

6-**9** were separated from Ex-4 and unreacted **1** on a C18 column (see Figure 3) monitored at both 280 and 360nm. A mobile phase of 0.1% TFA water was used with a flow rate of 1 mL/min and a gradient from 20-42.5% acetonitrile for 3 min then 42.5-47% acetonitrile for 12 min. Isolated conjugates **6**-**9** were

confirmed by electrospray mass spectrometry. The bioconjugates were separated as two peaks due to the aggregation of Ex-4.

5.3 In vitro work following agonism at GLP-1R, GIPR, NPY2R and NPY1R

5.3.1 GLP-1R agonism

HEK-293 cells stably transfected with human GLP-1R (HEK-GLP-1R) were used to test for agonism of each peptide and peptide conjugate. The HEK-GLP-1R cells were grown in DMEM with 10% fetal bovine serum (FBS), 1% pen-strep and 0.1% geneticin all produced by Gibco. Cells were incubated at 37 ^{o}C in a humidified incubator with 5% CO_2. Cells were plated at 60,000 cells per well on a 96 well plate coated with rat tail collagen and incubated for 24 h. To follow protein kinase A activity media was removed from the cells and DMEM containing 1% FBS and adenovirus incorporating the A-kinase activity reporter 3 (AKAR3) with a multiplicity of infection per cell of 25 was added to each well.[2] After 20 h of incubation the media was removed and 200 µL of standard extracellular solution (SES) was added to each well. Another 50 µL of SES containing conjugate was added to each well and immediately measured at 485 and 535 nm upon excitation at 440 nm. To follow cAMP production media was removed from the cells and DMEM containing RIP-CRE-Luc (200 ng/well), Lipofectamine, and Plus reagent was added to each well. Cells were incubated for 3 h and then an additional 200 µL of DMEM with 10% fetal bovine serum and 1% penstrep was added. After 36 of incubation the media was removed and 100 µL of DMEM with 0.1% bovine serum albumin containing the conjugate to be tested was added to each well and incubated for an additional 4 h. Cells were then lysed from the plate and tested for luciferase activity by measuring photoemissions.

5.3.2 GIPR agonism

HEK-293 cells stably transfected with human GIPR (HEK-GIPR) were used to test for agonism of **11**, **12**, **14**, and **15**. The HEK-GIPR cells were grown in DMEM with 10% fetal bovine serum (FBS), 1% pen-strep and 0.1% geneticin all produced by Gibco. Cells were incubated at 37 ^{o}C in a humidified incubator with

5% CO_2. Cells were plated at 60,000 cells per well on a 96 well plate coated with rat tail collagen and incubated for 24 h. To follow protein kinase A activity media was removed from the cells and DMEM containing 1% FBS and adenovirus incorporating the A-kinase activity reporter 3 (AKAR3) with a multiplicity of infection per cell of 25 was added to each well. After 20 h of incubation the media was removed and 200 µL of standard extracellular solution (SES) was added to each well. Another 50 µL of SES containing conjugate was added to each well and immediately measured at 485 and 535 nm upon excitation at 440 nm.

5.3.3 NPY1R and NPY2R agonism

CHO cells were used to test for agonism of each peptide and peptide conjugate. The CHO cells were grown in F12K media with 10% fetal bovine serum (FBS) and 1% pen-strep. Cells were incubated at 37 ^{o}C in a humidified incubator with 5% CO_2. Cells were plated at 20,000 cells per well on a 96 well plate coated with rat tail collagen and incubated for 24 h. To follow NPY2R or NPY1R activation, media was removed from the cells and F-12k containing either NPY2R or NPY1R (20 ng/well), Lipofectamine, and Plus reagent was added to each well. Cells were incubated for 3 h and an additional 200 µL of DMEM with 10% fetal bovine serum and 1% penstrep was added. After 36 of incubation the media was removed and 100 µL of SES containing Fura-2 AM and pluranic acid F-127 were added to the cells to follow Ca^{2+} production and incubated for 30 min. The SES mixture was removed and 150 µL of SES was added to each well. Another 50 µL of SES containing conjugate was added to each well and excited at both 355/9 nm and 375/9 nm. Upon Fura-2 coordination with Ca^{2+} 505 nm light was emitted.

5.4 In vivo studies following glucoregulation and food intake after 8 and 11 administrations in Sprague Dawley rats

In vivo studies on **8** were done in collaboration with the Hayes lab at UPenn, and in vivo studies on **11** were done in collaboration with the Roth lab at Seattle Children's Research Institute. In vivo studies are still ongoing for both **8** and **11**.

5.5 Glucoregulation Studies

Either saline, Ex-4 or 8 was administered at t=-30 min followed by an oral bolus of glucose at t=0 min. Blood glucose levels were monitored at t=-30, 0, 30, 60, and 120 min. Either saline, Ex-4 or 11 was administered at t=-30 min followed by an oral bolus of glucose at t=0 min. Blood glucose levels were monitored at t=-30, 0,5,15, 30, 60, and 120 min.

5.6 Food intake and body weight

Food intake was monitored using a metabolic cage. Food was removed from the rats during the last hour of the light cycle while the rats were weighed.

5.7 Pica study upon treatment with saline, Ex-4 or 8

Sprague Dawley rats were administered treatment and left alone in cages with both kaolin and chow present. Total kaolin consumption was measured after 24 h. A high consumption of kaolin is indicative of nausea.

5.8 References

1. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angewandte Chemie International Edition* **2001,** *40* (11), 2004-2021.
2. Allen, M. D.; Zhang, J., Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochemical and Biophysical Research Communications* **2006,** *348* (2), 716-721.

6 Future Work

6.1 Retest pica and perform conditioned taste aversion testing on rats administered 8

The pica study on rats after administration of **8** will be expended on to include a larger sample size and more accurate error bars. The rats will be administered either saline, Ex-4 or **8** and then exposed to kaolin again. Consumption of kaolin will be calculated and compared to baseline to determine relative levels of nausea caused by each treatment.

Nausea will also be measured through conditioned taste aversion (CTA). The rats will first be place under water depravation for one week and be allowed water for only a limited amount of time a day. After a week rats were given a flavored water solution during the period of water access. Right after the flavored water is taken away the rats are administered either vehicle or drug. This is done for a couple of days followed by resting period. Rats are then given a choice of flavored water and the position of the flavored waters is switched half way through the water period. A preference for the new flavor indicates CTA.[1]

6.2 Test for IF-8 protection against IF degrading proteases

IF-**8** will be screen against more proteases. Cathepsin L is known to degrade IF so the question that will be answered is whether IF will continue to protect B_{12} and **8** in the presence of cathepsin L.[2] I have shown that IF protects **8** from degradation relative to unbound controls in an environment where IF is resistant to the only proteases present. Upon internalization into the ileum IF is degraded by lysosomal protease cathepsin L. Will this environment also damage **8** or will IF offer enough protection to allow a high enough percentage of **8** to enter the blood stream intact.

The first part of this experiment will involve running control to determine if **8** is digested by cathepsin L. Once the level of degradation is known, a protease cocktail will be used to degrade IF-**8**. The protease mixture will first contain proteases used in the previously published work such as trypsin, chymotrypsin

and meprin β. Additional proteases will need to be tested especially other cathepsins. After establishing the robustness of IF-**8** protection other B_{12}-peptide conjugates will be tested for stability. Establishing IF binding as a way to improve protease resistance for a drug will open IF and B_{12} conjugation to investigation as a potentially new oral delivery technique.

6.3 Continue in vitro and in vivo testing on 11

6.3.1 Screen for activity at NPY1R and NPY2R following the native G_i pathway

An assay has been developed to follow the G_i pathway in NPY2R agonism. The current assay however does not produce a strong enough response to report an accurate dose response curve. HEK-293 cells endogenously express the adenosine receptor, which upon agonism causes an increase in cAMP levels.[3] A two injection system was used in preliminary data to first stimulate cAMP production through the G_s pathway, with an adenosine injection, and the second injection was used to shut down cAMP production through the G_i pathway using PYY(3-36). Figure 1 shows the ability of PYY(3-36) to lower cAMP levels and prevent FRET ratio from decreasing further.

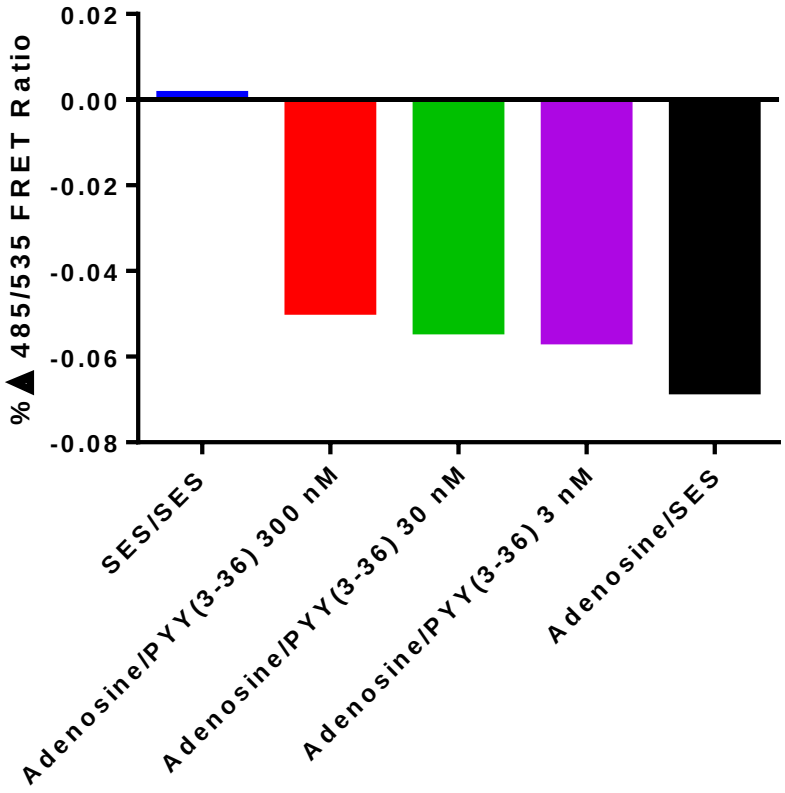


Figure 1. Adenosine induced cAMP rise to show PYY(3-360's ability to work through the Gi pathway and lower cAMP levels.

The raw data is shown as a ratio of 485/535 emission. The total reduction in is calculated by taking the total average FRET ratio change between 360 s and 420 s to determine the relative effectiveness of raising and lowering the cAMP and PKA levels.

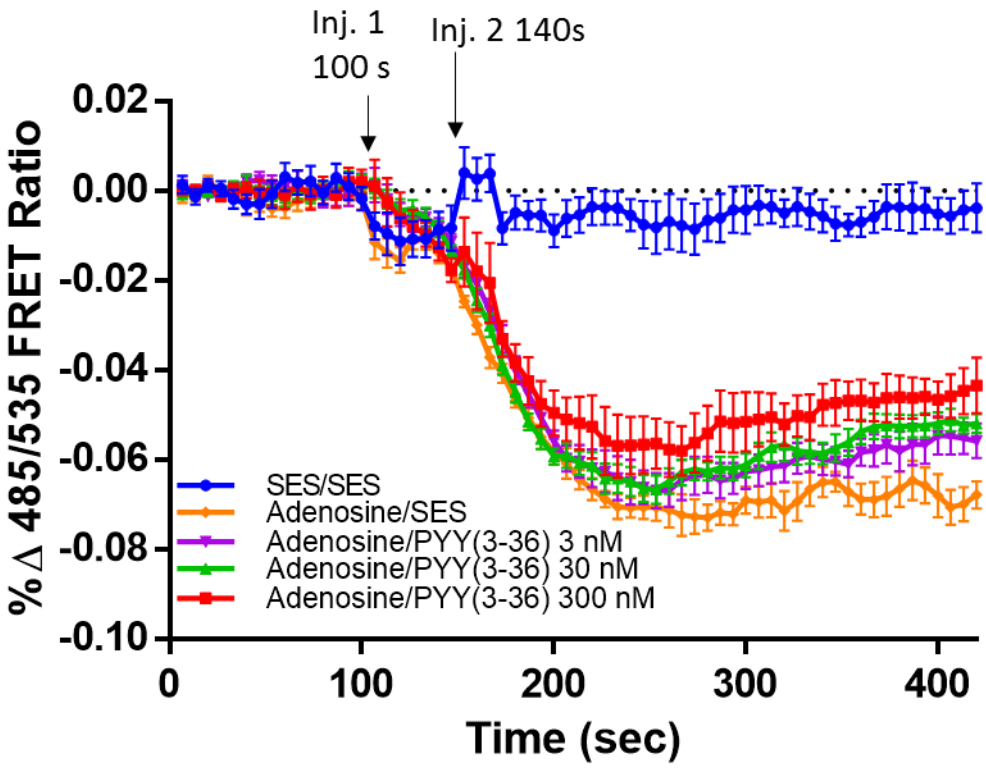


Figure 2. FRET ratio change in real time over 420s after a first injection of adenosine at 100 s followed by PYY(3-36) at 140 s.

Although the current assay to show native G_i pathway activation after NPY1R and NPY2R agonism does show a decrease in FRET ratio change the assay does not provide enough of a change to calculate a reliable dose response. This assay will have to be improved by either increasing cAMP levels or causing a greater difference in FRET ratio between the adenosine/SES and adenosine/PYY(3-36) samples. This can be done by modifying the assay in one or more of the following ways: optimizing receptor transfection, changing cell line, or changing the reporter.

6.3.2 Determine half-life of 11 in vivo

From chapter 4 I have shown that **11** has improved food intake reduction over Ex-4, but similar blood glucose control. One suspected reason for this is **11** has a longer half-life than Ex-4 causing appetite

suppressing effects to last longer. To test for this an ELISA will be used to test blood sample taken after administration of **11**. These samples will be analyzed for total in vivo residency and be used to calculate a half-life for **11**.

6.3.3 Modify 11 to improve upon the pharmacokinetic properties

Although **11** has improved weight loss capabilities when compared to Ex-4 it may be able to be further optimized. Additional sequence modifications to **11** could yield a more potent dual agonist or even triagonist. The in vivo effects of **11** are superior to Ex-4 and Ex-4/PYY(3-36) coadministration despite no evidence of NPY2R agonism and only slight agonism at the GIPR, when compared to native GIP. It is possible that GIPR agonism could be optimized through additional modifications. For this to be effective a binding study simulation between **11** and GIPR would need to be done.

Addition of PYY(3-36) amino acid sequence could alter **11** enough to add NPY2R agonism. **14** has a significantly greater portion of PYY(3-36) and work at nanomolar concentration in vitro although showed no food intake reduction in vivo. Glucagon receptor agonism may also be added through an amino acid modification of E3Q.[4]

6.3.4 Test 11 for pica and CTA response in rats

11 is similar to Ex-4 in amino acid sequence and glucose control but more potent at appetite suppressing. If this enhanced appetite suppressing ability is due to an increase in brain uptake and increased receptor agonism of GLP-1R in the AN, an increase in nausea may play a role in weight loss. To test for an increase in nausea a pica and CTA test will be run on rats administered **11.**

References

1. Kanoski, S. E.; Rupprecht, L. E.; Fortin, S. M.; De Jonghe, B. C.; Hayes, M. R., The role of nausea in food intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide. *Neuropharmacology* **2012,** *62* (5–6), 1916-1927.

2. Okuda, K., Discovery of vitamin B12 in the liver and its absorption factor in the stomach: A historical review. *Journal of Gastroenterology and Hepatology* **1999,** *14* (4), 301-308.

3. Cooper, J.; Hill, S. J.; Alexander, S. P., An endogenous A2B adenosine receptor coupled to cyclic AMP generation in human embryonic kidney (HEK 293) cells. *British journal of pharmacology* **1997,** *122* (3), 546-550.

4. Kosinski, J. R.; Hubert, J.; Carrington, P. E.; Chicchi, G. G.; Mu, J.; Miller, C.; Cao, J.; Bianchi, E.; Pessi, A.; SinhaRoy, R., The Glucagon Receptor Is Involved in Mediating the Body Weight‐Lowering Effects of Oxyntomodulin. *Obesity* **2012,** *20* (8), 1566-1571.

Ron L. Bonaccorso
310 Richfield Blvd Syracuse NY, 13211
rbonacco@syr.edu (585) 690-7438

Education

Syracuse University — Syracuse, NY
Ph.D. Chemistry — Expected 2016

Rochester Institute of Technology — Rochester, NY
B.S. Chemistry — 2012

I have been involved in research concerning many aspects of medicinal chemistry. I have been lead on projects that range from oral delivery of peptides to designing and optimizing novel peptides featuring dual agonism and/or biased agonism, primarily at G-coupled protein receptors. My research has focused on protein/peptide conjugation, with my interest lying in the use of the vitamin B12 dietary uptake pathway. My research has allowed me to pursue projects from concept to in vivo outcome (rodent models). My work has resulted in a paper in *Molecular Pharmaceutics* and two additional papers in the final in vivo stages of completion for publication in early 2017. Along with my advisor Professor Robert Doyle, I am co-author of a filed patent describing bi-agonists of both the Glucagon-like peptide-1 receptor and Neuropeptide Y2 receptor, aiming to both control glucose levels and facilitate food intake reduction concomitantly through a peptide pharmaceutical. My goal upon graduating in early summer 2016 is to continue to pursue research and development of in pharmaceuticals.

Skills

Designing mosaic peptides, GLP-1 and PYY assay design, G protein coupled receptor assay development for appetite and diabetes peptide drug development, fluorescent and bioluminescence plate assays, mammalian cell culture and cell transfection, plasmid isolation, site specific peptide/protein conjugation, click chemistry, organometallic synthesis, RP-HPLC, FPLC, (affinity, size-exclusion, IEC), MALDI-ToF-MS and ESI-MS, FTIR, 1- and 2-D NMR, western blot, fluorescence spectroscopy.

Research Experience

Graduate Research Assistant: **Syracuse University**
Worked in peptide conjugation, including purification and characterization (Syracuse University; advisor: Prof. Robert P. Doyle) along with cell culture and in vitro testing (SUNY Upstate collaborator, Prof. George G. Holz) and in vivo testing (Seattle Children's Research Institute; collaborator Prof. Christian L. Roth).

Undergraduate Research Technician: **Rochester Institute of Technology**
Designing an indicator to be used as a quick field test to check for counterfeit malaria medication in high risk countries.

Laboratory Technician: **University of Rochester Laboratory for Laser Energetics**
Synthesis and purification of ligands for inorganic dyes.

Awards

Syracuse University Travel Award — Summer 2013 – ACS Indianapolis; Fall 2015 – AAPS Orlando

Publications and Patents

- **Bonaccorso R. L.**, Chepurny O. G., Becker-Pauly C., Holz G. G., Doyle R. P., (2015). Enhanced Peptide Stability Against Protease Digestion Induced by Intrinsic Factor Binding of a Vitamin B12 Conjugate of Exendin-4 *Molecular Pharmaceutics* **12**(9): 3502-3506.

- Henry K. E., Kerwood D. J., Allis D. G., Workinger J., **Bonaccorso R. L.**, Holz G. G., Doyle R. P., Solution Structure and Constrained Molecular Dynamics Study of Vitamin B12 Conjugates of the Anorectic Peptide PYY(3-36) *ChemMedChem* **2016** DOI: 10.1002/cmdc.201600073.

- **Bonaccorso R. L.**, Doyle R. P., Coagonists of Glucagon-Like Peptide 1 Receptor and Neuropeptide Y2 Receptor *Patent Pending* SU100846

- **Bonaccorso R. L.**, Clinton E., Chepurny O. G., Holz G. G., Doyle R. P., Intrinsic Factor Binding of a Vitamin B12 Conjugate of Exendin-4 Shows Improved Glycemic Control in Rats *In preparation*

- **Bonaccorso R. L.**, Chepurny O. G., Holz G. G., Doyle R. P., Dual Agonism of the GLP-1 Receptor and NPY2-Receptor Through rational design of a Peptide Coagonist *In preparation*

Presentations and Posters

- **246th ACS National Meeting and Expo**, Indianapolis September 2013; *Poster*
- **Upstate Medical University**, Syracuse, NY, October 3, 2014; *Presentation*
- **Pfizer**, Cambridge, MA, November 21, 2014; *Invited Presentation*
- **ACS 2015 Northeast Regional Meeting**, Ithaca, NY, June 12, 2015; *Presentation*
- **AAPS National Conference Orlando** October 26, 2015; *Poster*
- **NYAS GLP-1 Treatment for Diabetes and Beyond** December 8, 2015; *Poster*

Professional Affiliations

- American Chemical Society (ACS)
- American Association of Pharmaceutical Scientists (AAPS)
- New York Academy of Sciences (NYAS)

Teaching Assistantships

Organic Chemistry I Lab, Fall 2012
Organic Chemistry II Lab, Spring 2013
General Chemistry II Recitation, Spring 2014
General Chemistry I Recitation, Fall 2014
General Chemistry II Lab, Spring 2015
General Chemistry I Recitation, Fall 2015
General Chemistry II Lab, Spring 2015

molecular pharmaceutics

Communication

pubs.acs.org/molecularpharmaceutics

Enhanced Peptide Stability Against Protease Digestion Induced by Intrinsic Factor Binding of a Vitamin B_{12} Conjugate of Exendin-4

Ron L. Bonaccorso,[†] Oleg G. Chepurny,[‡] Christoph Becker-Pauly,[∥] George G. Holz,[‡,§] and Robert P. Doyle*[,†,‡]

[†]Department of Chemistry, Center for Science and Technology, Syracuse University, 111 College Place, Syracuse, New York 13244, United States

[‡]Department of Medicine, State University of New York, Upstate Medical University, Syracuse, New York 13210, United States

[§]Department of Pharmacology, State University of New York, Upstate Medical University, Syracuse, New York 13210, United State

[∥]Institute of Biochemistry, Christian-Albrechts-University, 24118 Kiel, Germany

Supporting Information

ABSTRACT: Peptide digestion from proteases is a significant limitation in peptide therapeutic development. It has been hypothesized that the dietary pathway of vitamin B_{12} (B_{12}) may be exploited in this area, but an open question is whether B_{12}-peptide conjugates bound to the B_{12} gastric uptake protein intrinsic factor (IF) can provide any stability against proteases. Herein, we describe a new conjugate of B_{12} with the incretin peptide exendin 4 that demonstrates picomolar agonism of the glugacon-like peptide-1 receptor (GLP1-R). Stability studies reveal that Ex-4 is digested by pancreatic proteases trypsin and chymotrypsin and by the kidney endopeptidase meprin β. Prebinding the B_{12} conjugate to IF, however, resulted in up to a 4-fold greater activity of the B_{12}-Ex-4 conjugate relative to Ex-4, when the IF-B_{12}-Ex-4 complex was exposed to 22 μg/mL of trypsin, 2.3-fold greater activity when exposed to 1.25 μg/mL of chymotrypsin, and there was no decrease in function at up to 5 μg/mL of meprin β.

KEYWORDS: *vitamin B_{12}, exendin-4, intrinsic factor, trypsin, AKAR3*

INTRODUCTION

The human vitamin B_{12} (B_{12}) dietary uptake pathway is a complex process that facilitates access in humans to a vital cofactor of methionine synthase and methyl malonyl CoA mutase enzymes.[1] This pathway involves three major binders, two of which, intrinsic factor (IF) and haptocorrin (HC), being critical for oral uptake (the third, transcobalamin II (TCII), facilitates entry into cells upon enterocyte passage).[2] HC primarily protects B_{12} against acid digestion in the stomach and is enzymatically digested upon entry of the HC-B_{12} complex into the duodenum, whereupon the B_{12} is bound by IF. While IF is produced in gastric parietal cells and can bind B_{12} in the stomach, HC binding is preferred at the lower pH here and it is only upon digestion of HC and a rise in pH in the intestine that IF binding of B_{12} occurs naturally.[3,4] Concomitant with the rise in pH is the release of pancreatic proteases, and it is critical to note that IF, unlike HC, is resistant to pancreatic protease digestion.[5] IF is critical then for delivery of B_{12} through the intestinal tract to the ileum where cubilin-amnionless based receptor mediated enterocyte passage occurs.[6] Employing this pathway for oral peptide delivery, for example, requires conjugation of the peptide to B_{12} in such a way that IF recognition of B_{12} is not critically hindered and that B_{12} conjugated peptide can still exhibit the desired pharmacological function. Such concerns are typically readily addressed, however, and there are now several significant examples of B_{12}-peptide conjugates that meet the above criteria.[7−11] What is not understood, but is no less important, is whether such peptide function is maintained when the conjugate is bound to IF and whether IF, so effective at protecting B_{12}, can provide any protection to a B_{12} conjugated peptide upon exposure to a protease. To investigate these questions we decided to focus on a highly potent peptide (Ex-4) that is the basis of a pharmaceutical (exenatide) currently approved for treatment of diabetes mellitus.[12]

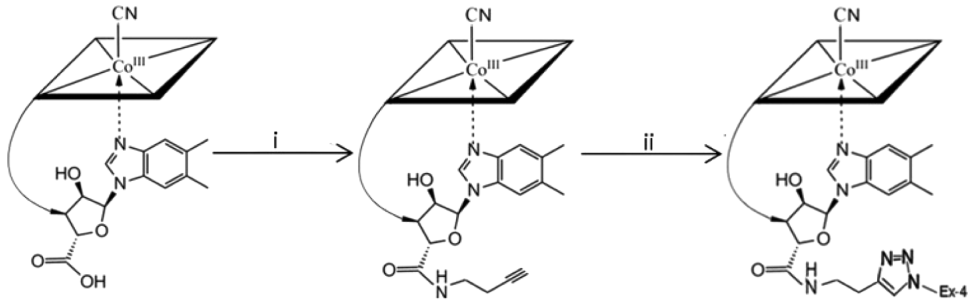
Ex-4 was discovered in the venom of the Gila monster in 1992 by Eng et al. and is an incretin mimetic, sharing 53% homology with glucagon-like peptide-1 (GLP-1). Like GLP-1, Ex-4 stimulates the release of insulin through agonism of the GLP-1 receptor (GLP-1R) (EC_{50} 33 pM), effectively lowering blood glucose levels. Unlike GLP-1, Ex-4 is resistant to the enzyme dipeptidyl peptidase IV (DPP-IV), which rapidly cleaves and inactivates GLP-1 in vivo.[13,14] Since DPP-IV cleaves any peptide with an alanine or proline at the second position from the N-terminus, substituting a glycine for the alanine in GLP-1 results in the resistance seen in Ex-4. This resistance allows Ex-4 to have a half-life of 2.4 h compared to <2 min as seen for GLP-1.[15] Such resistance to DPP-IV does not, however, translate to other proteases, and exenatide therefore must be administered subcutaneously.

The hypothesis herein then is that this pancreatic degradation and general protease limitation may be overcome, at least to some degree above unmodified peptide, by conjugating B_{12} to Ex-4 and subsequently adding IF, assuming the necessary maintenance of B_{12} binding by IF and Ex-4 agonism are controlled. To test these hypotheses we

Received: May 19, 2015
Revised: July 6, 2015
Accepted: August 5, 2015
Published: August 11, 2015

DOI: 10.1021/acs.molpharmaceut.5b00390
Mol. Pharmaceutics 2015, 12, 3502−3506

Scheme 1. Synthesis of B_{12}-Ex-4 Conjugate 4[a]



2 3 4

[a]Reagents and conditions: (i) EDCI, HOBt, 1-amino-3-butyne, rt, 16 h; (ii) **1**, $CuSO_4$, sodium ascorbate, 1 h.

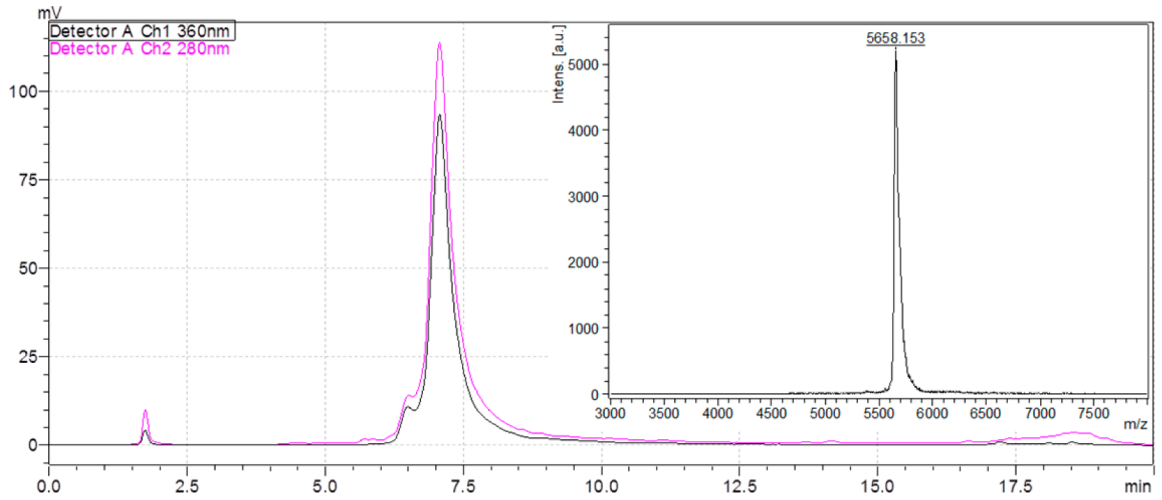


Figure 1. LC trace showing purified **4** as a monomer (~7 min) and dimer (~6.5 min) and MALDI-Tof MS (inset) of **4** showing m/z of 5658.153 Da, which corresponds to the +1 of **4**.

synthesized a B_{12}-Ex-4 conjugate focusing on the lysine 12 (K12) position of Ex-4 and the ribose 5′-hydroxyl group of the B_{12} moiety as sites of conjugation since both sites on the respective moieties had published precedent for allowable modification.[16−18] Binding to IF was confirmed by radioassay, and agonism of the GLP-1 receptor was then established for an azido modified K12-Ex-4 (**1**), B_{12}-Ex-4 (**4**), and IF-B_{12}-Ex-4 (IF-**4**). With such establishing parameters controlled for stability against the abundant intestinal endopeptidases, trypsin, chymotrypsin, and the kidney protease meprin β[19] were compared for **1**, **4**, and IF-**4**.

EXPERIMENTAL SECTION

(AzidoK12)-Ex-4 (**1**) was conjugated to B_{12} at the K12 position using Huisgens/Sharpless click chemistry,[20] using Ex-4 modified at the lysine 12 ε-amine with an azido group during solid-phase synthesis. The 5′ hydroxyl group of B_{12} was also modified prior to coupling, being selectively oxidized to a carboxylic acid (**2**) using 2-iodoxybenzoic acid, as previously described by us.[21] Subsequent coupling of 1-amino-3-butyne to **2** with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDCI) and 1-hydroxybenzo-triazole (HOBt) produced B_{12} with a terminal alkyne at the ribose 5′-position (**3**) (see Scheme 1). Compound **3** was purified using a Shimadzu Prominence HPLC on an Eclipse XDB C18 5 μm 4.6 mm × 150 mm column with a mobile phase of 0.1% TFA water and elution with acetonitrile on a gradient starting at 15% acetonitrile increasing to 35% over 20 min (NMR for **3** is provided as Supplementary Figure S1).

Compounds **1** and **3** were coupled using copper(II) sulfate and sodium L-ascorbate (see Scheme 1). The new B_{12}-Ex-4 conjugate (**4**) was purified with a Shimadzu HPLC using an Eclipse XDB C18 5 μm 4.6 mm × 150 mm column with a mobile phase of 0.1% TFA water and elution with acetonitrile. A gradient run from 20% acetonitrile to 42% acetonitrile during the first 3 min and then 42 to 47.5% acetonitrile during the next 10 min was used to separate **4** from starting materials. The product was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-ToF MS) (see Figure 1, inset). Compound **4** was purified to greater than 97% purity by HPLC (see Figure 1). The tendency of Ex-4 to aggregate resulted in a small shoulder at 6.5 min.[22]

DISCUSSION

Initially, IF binding of **4** was confirmed by radiometric chase assay using ^{57}Co-labeled B_{12} and compared to free B_{12}, as cyanocobalamin (see Figure 2).[23] Significant IF binding of **4** (6.8 nM) was maintained, albeit reduced from unmodified B_{12} (0.12 nM).

Once IF binding of **4** (IF-**4**) was confirmed, agonism of the GLP-1R was assayed for **1**, **4**, and IF-**4** using HEK-293 cells stably transfected with the GLP-1R (HEK-GLP-1R).[25] To this

DOI: 10.1021/acs.molpharmaceut.5b00390
Mol. Pharmaceutics 2015, 12, 3502−3506

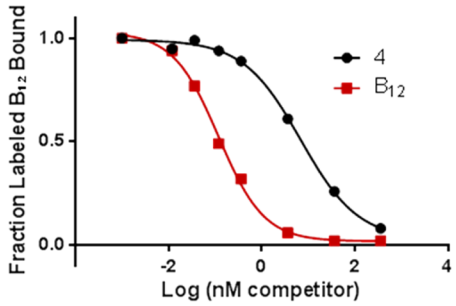


Figure 2. IF binding to B_{12} (0.12 nM) and **4** (6.8 nM). IF used in these assays was produced in the plant *Arabidopsis* in the apo-form and of high purity.[24]

end, we employed a new assay that uses adenoviral transduction to express the genetically encoded FRET reporter AKAR3 that serves as a sensitive readout for cAMP production due to the fact that AKAR3 undergoes a decrease of 485/535 nm emission FRET ratio when it is phosphorylated by cAMP-dependent protein kinase A (PKA) subsequent to GLP-1R activation.[26−28] This is the first instance to our knowledge of a FRET assay for GLP-1R using viral AKAR3 and offers a ready route to sensitive high-throughput screening of the GLP-1R. An EC_{50} for **1**, **4**, and IF-**4** were measured at 26, 68, and 132 pM, respectively (see Figure 3). It is worth noting that the azido modification to

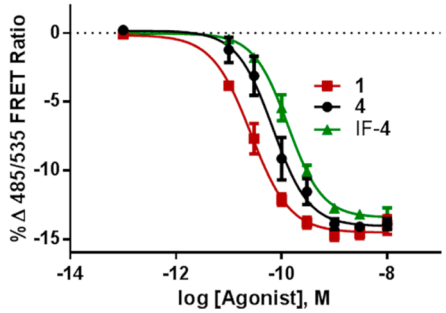


Figure 3. Dose−response analysis of **1**, **4**, and IF-**4** yielded EC_{50} values of 26, 68, and 132 pM, respectively, as determined by monitoring the 485/535 nm FRET emission ratio.

the K12 position of Ex-4 showed no significant reduction in potency compared to unmodified Ex-4 suggesting a useful general route for selective conjugation to Ex-4 through click chemistry approaches.[20] Compounds **4** and IF-**4** show that further conjugation to the K12 position effects function but still demonstrates low picomolar effective concentrations.

Compounds **1**, **4**, and IF-**4** were analyzed for stability against proteolysis by measuring remaining function at the receptor compared to undigested controls. Compounds **1**, **4**, and IF-**4** were tested for function at [100 nM], a concentration at which each had comparable percent change in FRET ratio (see Table 1). Each protease was analyzed separately so that the protective nature of B_{12} and IF could be analyzed for their effect versus the specific protease. The pH sensitivity of the assay prevented the use of actual intestinal fluids when testing the compounds.

Table 1. Percent Change in FRET at 100 nM for 1, 4, and IF-4

compd	% change in FRET at 100 nM
1	−12 ± 0.01
4	−13 ± 0.02
IF-4	−12 ± 0.01

Digestion was conducted in a standard extracellular solution containing trypsin at 11, 22, or 50 μg/mL, chymotrypsin at 1.25, 3, or 6.25 μg/mL, or meprin β at 1 or 5 μg/mL (see Figures 4 and S2).

At the lowest concentrations of trypsin (11 μg/mL) and chymotrypsin (1.25 μg/mL) there is up to 50% greater function for IF-**4** relative to **4** alone with the highest concentration of trypsin (50 μg/mL) and chymotrypsin (6.25 μg/mL) assayed showing complete lack of function for all systems. The digestion was monitored by measuring agonism of the drugs at the GLP-1R, initially over the course of 3 h, although it was quickly noted that there was no change after 1.5 h indicating that the digestion had stopped by this time point (data not shown). Subsequent triplicate runs were then performed on digestions of 1.5 h.

Meprin β digestion revealed a 2-fold increase in function with B_{12} conjugation and a 3-fold increase in function when prebound to IF (see Figures 5 and S3). No function was seen for **1** at concentrations greater than 3 μg/mL. The protection provided from B_{12} conjugation and subsequent binding to IF show that key residues are being protected. Results of the AKAR3 assays show maintenance of function where otherwise none was observed or improvement of function when **4** is first bound to IF.

CONCLUSIONS

The conservation or improved relative function demonstrated herein for Ex-4 when conjugated to B_{12}, and more significantly when bound by IF, is an important first-step in addressing the use and putative role of IF in protecting an administered peptide (orally or by injected means). Protection against pancreatic protease-catalyzed hydrolytic digestion of **4** was maximal at a trypsin concentration of 22 μg/mL and 3 μg/mL of chymotrypsin when **4** was prebound to IF, providing a 4-fold and 5-fold positive increase in function, respectively, as measured by GLP-1R agonism (utilizing the AKAR3 screening assay). The digestion with metalloendoprotease meprin β showed the most significant protection when comparing **1** and IF-**4**. No reduction in function was seen at the highest concentration of meprin β tested (5 μg/mL), while **1** showed no function at concentrations greater than 1 μg/mL of meprin β. B_{12} provided some protection against trypsin relative to the native peptide. The effect is seen at 11 μg/mL of trypsin with a relative 4-fold increase and at 1.25 and 3 μg/mL of chymotrypsin with a relative increase of 3- and 5-fold. The fact that the IF bound form IF-**4** still maintained significant function at the GLP-1R is also highly significant since many routes to protect against intestinal degradation involve encapsulation, which prevents possible luminal function or absorption when in place. The use of IF to improve the protease stability of a peptide offers significant scope for exploitation. Even a small improvement in oral function, for example, may be sufficient to achieve the desired effect. Combining this approach with a highly potent peptide with known gut receptors that can produce a vagal afferent response (such as, but not limited to, GLP-1/Ex-4 or PYY3-36), for example, may allow for a positive clinical outcome to be achieved orally, without need even for systemic delivery. Finally, as demonstrated by the stability against meprin β, there is no suggestion that this approach is limited to oral use against

DOI: 10.1021/acs.molpharmaceut.5b00390
Mol. Pharmaceutics 2015, 12, 3502−3506

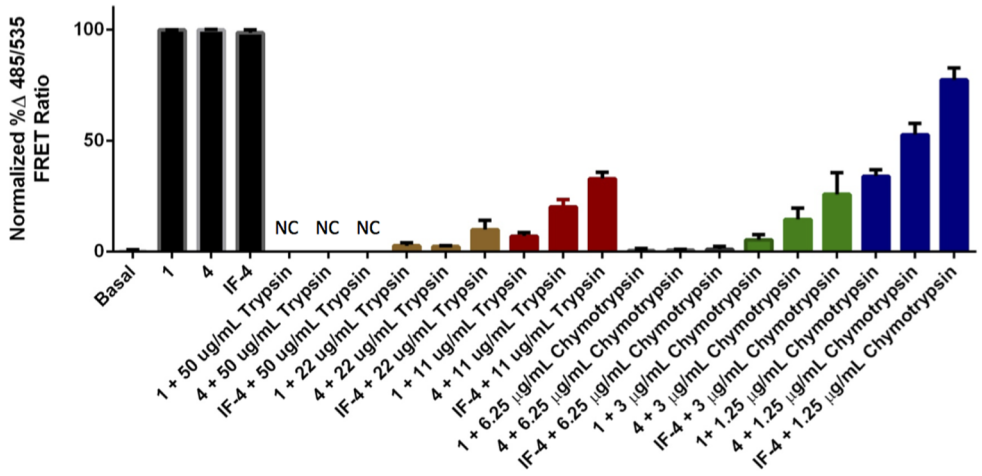


Figure 4. Digestion for 1.5 h of 100 nM **1**, **4**, and IF-**4** with 50, 22, or 11 μg/mL of trypsin or 1.25, 3, or 6.25 μg/mL of chymotrypsin using AKAR3 to measure function. The data shows the maximum expression normalized to 100% of the conjugates done in triplicate (mean ± SEM). Basal control contained trypsin at 50 μg/mL of trypsin. (N.C. = no change). A scatterplot analysis is provided in the Supporting Information (Figure S2).

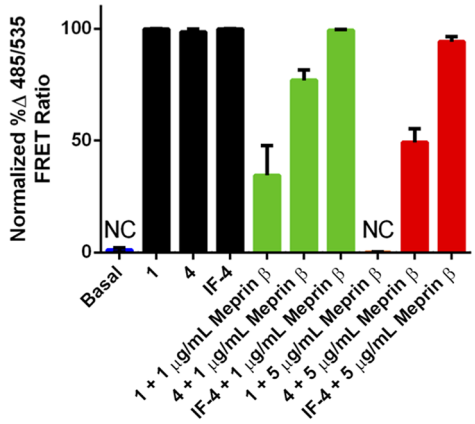


Figure 5. Thirty minute meprin β digestion of 100 nM **1**, **4** and IF-**4** with 1 and 5 μg/mL of meprin β. The data shows the maximum expression normalized to 100% of the conjugates done in triplicate (mean ± SEM). Basal control contained 2 μg/mL of meprin β. Recombinant human meprin β was produced in insect cells and purified and activated as described previously.[29] A scatterplot analysis is provided in the Supporting Information (Figure S3).

gastric proteases, but could also be expanded into serum (through subcutaneous or intravenous injection of IF bound B_{12}-peptide conjugates, for instance), facilitating greatly improved pharmacokinetics (especially when combined with prior results showing B_{12} conjugation already improved sc absorption of a PYY3-36 conjugate[8]), making this a possible platform technology for peptide drug development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00390.

NMR data and scatterplot analyses (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rpdoyle@syr.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the lab of Prof. Ebba Nexo (Department of Clinical Medicine−Clinical Biochemistry, University of Aarhus, Denmark) for performing the IF binding assays[23] and Prof. C. Becker-Pauly for providing meprin β produced with support of the German Research Foundation (DFG) Grant SFB877. R.P.D. acknowledges Xeragenx LLC (St. Louis, MO, USA; Xeragenx.com) for research funding and for providing intrinsic factor (#XGX-003).

■ REFERENCES

(1) Banerjee, R. *Chemistry and Biochemistry of B12*; John Wiley & Sons: New York, 1999.
(2) Nielsen, M. J.; Rasmussen, M. R.; Andersen, C. B. F.; Nexo, E.; Moestrup, S. K. Vitamin B12 transport from food to the body's cells-a sophisticated, multistep pathway. *Nat. Rev. Gastroenterol. Hepatol.* **2012**, *9* (6), 345−354.
(3) Gräsbeck, R. Hooked to vitamin B12 since 1955: A historical perspective. *Biochimie* **2013**, *95* (5), 970−975.
(4) Alpers, D. H.; Russell-Jones, G. Gastric intrinsic factor: The gastric and small intestinal stages of cobalamin absorption. A personal journey. *Biochimie* **2013**, *95* (5), 989−994.
(5) Allen, R. H.; Seetharam, B.; Podell, E.; Alpers, D. H. Effect of Proteolytic Enzymes on the Binding of Cobalamin to R Protein and Intrinsic Factor: In Vitro Evidence That A Failure To Partially Degrader Protein Is Responsible For Cobalamin Malabsorption In Pancreatic Insufficiency. *J. Clin. Invest.* **1978**, *61* (1), 47−54.
(6) Gherasim, C.; Lofgren, M.; Banerjee, R. Navigating the B12 Road: Assimilation, Delivery, and Disorders of Cobalamin. *J. Biol. Chem.* **2013**, *288* (19), 13186−13193.
(7) Clardy-James, S.; Chepurny, O. G.; Leech, C. A.; Holz, G. G.; Doyle, R. P. Synthesis, Characterization and Pharmacodynamics of Vitamin-B(12)-Conjugated Glucagon-Like Peptide-1. *ChemMedChem* **2013**, *8* (4), 582−586.
(8) Henry, K. E.; Elfers, C. T.; Burke, R. M.; Chepurny, O. G.; Holz, G. G.; Blevins, J. E.; Roth, C. L.; Doyle, R. P. Vitamin B12 Conjugation of Peptide-YY3−36 Decreases Food Intake Compared to Native Peptide-YY3−36 Upon Subcutaneous Administration in Male Rats. *Endocrinology* **2015**, *156* (5), 1739−1749.
(9) Fazen, C. H.; Valentin, D.; Fairchild, T. J.; Doyle, R. P. Oral delivery of the appetite suppressing peptide hPYY(3−36) through the vitamin B12 uptake pathway. *J. Med. Chem.* **2011**, *54*, 8707−8711.

(10) Chalasani, K. B.; Russell-Jones, G. J.; Jain, A. K.; Diwan, P. V.; Jain, S. K. Effective oral delivery of insulin in animal models using vitamin B12-coated dextran nanoparticles. *J. Controlled Release* **2007**, *122* (2), 141−150.
(11) Siega, P.; Wuerges, J.; Arena, F.; Gianolio, E.; Fedosov, S. N.; Dreos, R.; Geremia, S.; Aime, S.; Randaccio, L. Release of Toxic Gd3+ Ions to Tumour Cells by Vitamin B12 Bioconjugates. *Chem. - Eur. J.* **2009**, *15* (32), 7980−7989.
(12) Eng, J.; Kleinman, W. A.; Singh, L.; Singh, G.; Raufman, J. P. Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* **1992**, *267* (11), 7402−5.
(13) Mentlein, R.; Gallwitz, B.; Schmidt, W. E. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7−36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur. J. Biochem.* **1993**, *214* (3), 829−835.
(14) Nielsen, L. L.; Young, A. A.; Parkes, D. G. Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycemic control of type 2 diabetes. *Regul. Pept.* **2004**, *117* (2), 77−88.
(15) Schnabel, C. A.; Wintle, M.; Kolterman, O. Metabolic Effects of the Incretin Mimetic Exenatide in the Treatment of Type 2 Diabetes. *Vascular Health and Risk Management* **2006**, *2* (1), 69−77.
(16) Clardy, S. M.; Allis, D. G.; Fairchild, T. J.; Doyle, R. P. Vitamin B12 in drug delivery: breaking through the barriers to a B12 bioconjugate pharmaceutical. *Expert Opin. Drug Delivery* **2011**, *8* (1), 127−140.
(17) Son, S.; Chae, S. Y.; Kim, C. W.; Choi, Y. G.; Jung, S. Y.; Lee, S.; Lee, K. C. Preparation and Structural, Biochemical, and Pharmaceutical Characterizations of Bile Acid-Modified Long-Acting Exendin-4 Derivatives. *J. Med. Chem.* **2009**, *52* (21), 6889−6896.
(18) Jin, C.-H.; Chae, S. Y.; Son, S.; Kim, T. H.; Um, K. A.; Youn, Y. S.; Lee, S.; Lee, K. C. A new orally available glucagon-like peptide-1 receptor agonist, biotinylated exendin-4, displays improved hypoglycemic effects in db/db mice. *J. Controlled Release* **2009**, *133* (3), 172−177.
(19) Broder, C.; Becker-Pauly, C. The metalloproteases meprin α and meprin β: unique enzymes in inflammation, neurodegeneration, cancer and fibrosis. *Biochem. J.* **2013**, *450* (2), 253−264.
(20) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chem., Int. Ed.* **2001**, *40* (11), 2004−2021.
(21) Clardy-James, S. M.; Bernstein, J. L.; Kerwood, D. J.; Doyle, R. P. Site-Selective Oxidation of Vitamin B12 Using 2-Iodoxybenzoic Acid. *Synlett* **2012**, *23*, 2363−2366.
(22) Andersen, N. H.; Brodsky, Y.; Neidigh, J. W.; Prickett, K. S. Medium-Dependence of the secondary structure of exendin-4 and glucagon-like-peptide-1. *Bioorg. Med. Chem.* **2002**, *10* (1), 79−85.
(23) Stupperich, E.; Nexo, E. Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin B12 binding proteins intrinsic factor, transcobalamin and haptocorrin. *Eur. J. Biochem.* **1991**, *199* (2), 299−303.
(24) Fedosov, S. N.; Laursen, N. B.; Nexo, E.; Moestrup, S. K.; Petersen, T. E.; Jensen, E. Ø.; Berglund, L. Human intrinsic factor expressed in the plant Arabidopsis thaliana. *Eur. J. Biochem.* **2003**, *270* (16), 3362−3367.
(25) Gromada, J.; Rorsman, P.; Dissing, S.; Wulff, B. S. Stimulation of cloned human glucagon-like peptide 1 receptor expressed in HEK 293 cells induces cAMP-dependent activation of calcium-induced calcium release. *FEBS Lett.* **1995**, *373* (2), 182−186.
(26) Allen, M. D.; Zhang, J. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem. Biophys. Res. Commun.* **2006**, *348* (2), 716−721.
(27) Chepurny, O. G.; Kelley, G. G.; Dzhura, I.; Leech, C. A.; Roe, M. W.; Dzhura, E.; Li, X.; Schwede, F.; Genieser, H.-G.; Holz, G. G. *PKA-dependent potentiation of glucose-stimulated insulin secretion by Epac activator 8-pCPT-2-O-Me-cAMP-AM in human islets of Langerhans. Am. J. Physiol. Endocrinol. Metabol* **2010**, *298*, E622−E633.
(28) Holz, G. G.; Chepurny, O. G.; Leech, C. A.; Roe, M. W. High-throughput FRET assays for fast time-dependent detection of cyclic AMP in pancreatic beta cells. In *Cyclic Nucleotide Signaling*; Xiaodong, C., Ed.; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2015; pp 35−59.
(29) Becker, C.; Kruse, M. N.; Slotty, K. A.; Köhler, D.; Harris, J. R.; Rösmann, S.; Sterchi, E. E.; Stöcker, W. Differences in the Activation Mechanism between the α and β Subunits of Human Meprin. *Biol. Chem.* **2003**, *384*, 825−83.

DOI: 10.1021/acs.molpharmaceut.5b00390
Mol. Pharmaceutics 2015, 12, 3502−3506

Solution Structure and Constrained Molecular Dynamics Study of Vitamin B_{12} Conjugates of the Anorectic Peptide PYY(3–36)

Kelly E. Henry,[a] Deborah J. Kerwood,[a] Damian G. Allis,[a] Jayme L. Workinger,[a] Ron L. Bonaccorso,[a] George G. Holz,[b] Christian L. Roth,[c] Jon Zubieta,[a] and Robert P. Doyle*[a, b]

Vitamin B_{12}–peptide conjugates have considerable therapeutic potential through improved pharmacokinetic and/or pharmacodynamic properties imparted on the peptide upon covalent attachment to vitamin B_{12} (B_{12}). There remains a lack of structural studies investigating the effects of B_{12} conjugation on peptide secondary structure. Determining the solution structure of a B_{12}–peptide conjugate or conjugates and measuring functions of the conjugate(s) at the target peptide receptor may offer considerable insight concerning the future design of fully optimized conjugates. This methodology is especially useful in tandem with constrained molecular dynamics (MD) studies, such that predictions may be made about conjugates not yet synthesized. Focusing on two B_{12} conjugates of the anorectic peptide PYY(3–36), one of which was previously demonstrated to have improved food intake reduction compared with PYY(3–36), we performed NMR structural analyses and used the information to conduct MD simulations. The study provides rare structural insight into vitamin B_{12} conjugates and validates the fact that B_{12} can be conjugated to a peptide without markedly affecting peptide secondary structure.

Introduction

Peptide YY (PYY), a member of the pancreatic polypeptide family,[1–8] was first isolated from porcine intestinal tissue extracts in 1980[9] and was later shown to be a critical enteroendocrine hormone involved in appetite regulation.[10–12] PYY has two main circulating forms: PYY(1–36) and a truncated form, PYY(3–36).[13] PYY(1–36) is released in concert with caloric intake or exercise and is cleaved by dipeptidyl peptidase IV (DPP-IV)[14] in the gut to produce PYY(3–36). PYY(1–36) has an appetite-stimulating effect through activation of the orexigenic Y1 receptor (Y1-R) located in the intestines, blood vessels, and brain.[15] The two-amino-acid N-terminal (Tyr-Pro) truncation to PYY(3–36) results in an approximate 100-fold decrease in activity at the Y1-R,[5,16] and generates an agonist of the anorexigenic Y2 receptor (Y2-R) located in the intestines (vagal afferent sensory neuron signaling)[17,18] and brain,[19,20] which exerts a G-protein-coupled receptor (GPCR) G_i-mediated anorectic effect.

We recently reported a B_{12}–PYY(3–36) conjugate that demonstrated similar activity to native PYY(3–36) at the Y2-R in vitro, but improved function over PYY(3–36) upon subcutaneous (s.c.) administration in vivo in a lean rat model (conjugate **3** as described herein below).[21] In earlier work, we focused on conjugating to B_{12} through the ribose hydroxy group, as it is well established in the field that this is an optimal site for such conjugation, as it does not hinder recognition of B_{12} by its carrier proteins.[22–25] Likewise, we focused on an N-terminal region of PYY(3–36) for conjugation (specifically the K_4 residue), because again it has been well documented that modifications at (or indeed complete loss of) this area do not significantly affect Y2-R agonism.[26–28] These assumptions bore out, as the EC_{50} values at the Y2-R obtained for the conjugate produced (and noted as conjugate **3** herein) were similar to that of unconjugated PYY(3–36) used for comparison. These were established using a Fura-2 assay that monitors intracellular Ca^{2+} mobilization under conditions in which the Y2-R signals through a promiscuous G_q GTP binding protein.[21] Questions that remained from this work, however, were what affect, if any, does B_{12} conjugation actually have on the PYY(3–36) secondary structure and whether MD simulations could be used to better understand, and possibly predict, any structural modifications observed. To investigate these questions, two conjugates located at the same coupling sites (ribose on B_{12} and K_4 on PYY(3–36)

[a] Dr. K. E. Henry, Dr. D. J. Kerwood, Dr. D. G. Allis, J. L. Workinger, R. L. Bonaccorso, Prof. J. Zubieta, Prof. R. P. Doyle
Department of Chemistry, Center for Science and Technology
Syracuse University, 111 College Place, Syracuse, NY 13244 (USA)
E-mail: rpdoyle@syr.edu

[b] Prof. G. G. Holz, Prof. R. P. Doyle
Department of Medicine and Pharmacology
Institute for Human Performance, SUNY Upstate Medical University
750 East Adams Street, Syracuse, NY 13210 (USA)

[c] Prof. C. L. Roth
Department of Pediatrics, University of Washington, Division of Endocrinology and Diabetes, Seattle Children's Research Institute, Center for Integrative Brain Research, 1900 Ninth Avenue, Seattle, WA 98101 (USA)

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/cmdc.201600073.

but with a slightly varying spacer length (one methylene unit difference) were assayed for agonism of the Y2-R using a new fluorescence resonance energy transfer (FRET)-based assay that faithfully reports the normal signal transduction process by which the Y2-R signals through G_i proteins to lower levels of intracellular cyclic adenosine monophosphate (cAMP).[29] NMR structures were solved for these conjugates, and their in-solution NMR structures were compared with those previously reported by Keire et al.[30] and Nygaard et al.[7] Subsequent unconstrained and NMR constrained MD simulations were then also performed.

Results and Discussion

In vitro evaluation of conjugates 3 and 4

All assays were performed *at least* in triplicate. Conjugates **3** and **4** were tested for their abilities to lower cAMP levels. Figure 1 shows the dose–response relationships for **3** and **4** relative to K_4PYY, showing both conjugates are less active than K_4PYY and that **4** is more active than **3**. Both conjugates are, however, within one-half log order of K_4PYY.

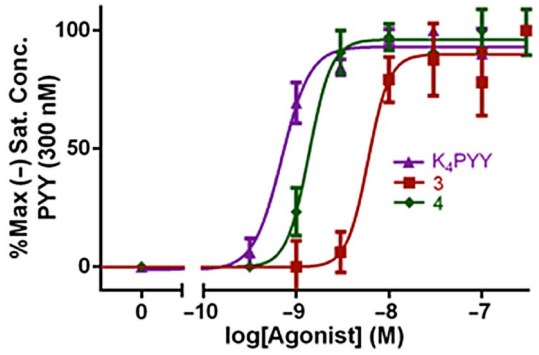


Figure 1. Dose–response curves monitored as percent inhibition of Ex-4 action at AKAR3 by K_4PYY and conjugates **3** and **4** at the Y2-R. EC_{50} values: K_4PYY: 1±0.2 nM, **3**: 6±2 nM, **4**: 2±0.2 nM. EC_{50} values are the average ±SEM.

This difference in EC_{50} values in comparing conjugates with K_4PYY was initially presumed to be a compromise between steric hindrance and/or unfavorable flexibility resulting in modifications/interactions that negatively affect the peptide structure–activity profile. At this point we decided to pursue NMR and molecular dynamics (MD) studies to further explore these in vitro observations.

NMR analysis of 3 and 4

Proton chemical shifts for **3** were assigned by analyzing TOCSY, DQF-COSY, and 2D NOE spectra at 25 °C. The experiments were also performed at 20 and 30 °C, at which slight shifts of some proton signals resolved overlapping peaks. The NMR spectra of **3** have some similarity to those of PYY(3–36), such as line broadening of many signals,[7] indicating increased dynamics, and a decrease in the chemical shift range of the backbone amide protons relative to full-length PYY. All backbone amide protons were assigned with the exception of Leu24, which could not be definitely determined due to overlap. Figure S17 (Supporting Information) shows the proton chemical shift difference between **3** and PYY(3–36). There are chemical shift differences for residue Lys4, which is not surprising, as this is the attachment site for B_{12}. Figure 2 shows an overlay of **3** after

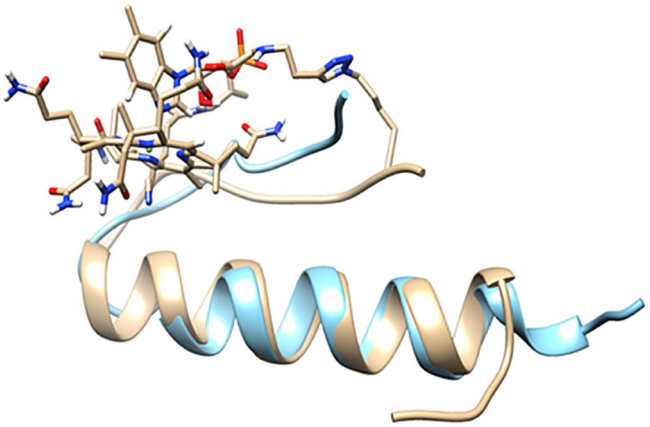


Figure 2. Overlay of PYY(3–36) (blue) versus **3** (tan). The structure of PYY(3–36) was obtained from the RCSB Protein Data Bank (www.pdb.org); PDB ID: 2DF0. The program Chimera (UCSF; www.cgl.ucsf.edu/chimera/)[31] was used to display the image.

MD calculations and PYY(3–36). The first chemical shift difference to consider is that for the methyl and α protons of Ala7. In PYY(3–36), the methyl group of residue Ala7 is pointing toward the α helix, whereas in conjugate **3** the methyl group is oriented away from the α helix. A 2D NOE cross-peak between the Ala7 methyl group and the ring protons of Tyr20 was observed in PYY(3–36),[7] but in **3** only a very weak cross-peak was observed in the longest mixing time 2D NOE experiment. This difference in orientation would also put the Ala7 α proton in distinct environments. The backbone amide protons have different chemical shifts for the β turn residues and residues in the N-terminal side of the α helix, specifically residues Gly9, Ala12, Ser13, Glu15, Leu17, and Arg19. This can be explained by the unraveling of the α helix and increased dynamics at the N-terminal side in the PYY(3–36) structure. Conjugate **3** maintains an α-helical structure similar to that of full-length PYY, and the residue with the largest chemical shift difference for the amide proton, Leu17, has a chemical shift value closer to that of the full-length peptide: PYY Leu17 NH 8.40 ppm,[7] **3** Leu17 NH 8.36 ppm, PYY(3–36) 7.95 ppm.[7] Nygaard et al. suggest that the Pro2–Tyr27 interaction is important for the stability of the PP fold, and that loss of this interaction in PYY(3–36) creates both conformational and dynamic changes in the structure, especially around the turn region.[7] Close inspection of **3** indicates possible hydrogen bonds from Glu6 to Tyr27 and Ser23, which may stabilize the PP fold in the conjugate.[7]

To elaborate on the structural studies, we decided to also investigate the solution structure of **4** to allow a direct comparison with **3**. Based on the NMR spectra, the conjugate structures appear very similar with only minor differences in the proton chemical shift assignments (Supporting Information Figure S18) and greater than 95% similarity in the 2D NOE spectra (Supporting Information Figure S19).

The major difference observed between **3** and **4** is the proton chemical shift change at B7 and B2 on the dimethylbenzimidazole (DMB) ligand (see Supporting Information Figure S12 for B_{12} atom numbering scheme). The C20 methyl protons are closer to the B4 and B2 protons in **4** based on the presence of a weak cross-peak between the C20 methyl protons and B2, which is only seen in the longest mixing time 2D NOE spectra of **3**, and a cross-peak between the methyl protons and B4, which is stronger for **4**. Weak cross-peaks are observed in **4** between a propionamide proton of the *g* side chain of B_{12} (Supporting Information Figure S12) and the Hα of D11 as well as the methyl protons of A12. The α helix motif as a whole is critical for association and subsequent agonism.[26] This is consistent with reported Y2-R interactions, as the C-terminal pentapeptide region is well established as the critical region, or "address", of the main interactions with Y2-R, while the α helix is considered the "message", indicating that both areas are critical in Y2-R agonism.[26]

NMR constrained MD studies of 3 and 4

Molecular dynamics simulations of **3** and **4** were performed both with and without the NMR constraints defined for **3** to consider differences in behavior and potential alternative structures in the simulations. The observed structural changes across the simulations identified intra-PYY(3–36) interactions that might, through their stabilization in the isolated conjugate, promote the decreased activity of **3** relative to **4**. The unconstrained MD simulation data then provide an additional set of structures for considering accessible geometries beyond the restrained set. The average structures from representative MD time ranges for **3** and **4** are shown in Figures 3 (front/side view) and 4 (top view). Apparent from these views, and the full simulations in general, are the persistence of 1) much of the α helical structure and 2) localization of the B_{12} fragment itself to the unstructured region approaching the loop into the α helix (in the images, this loop is at the base of all structures, including residues Glu10, Asp11, and Ala12). Across all of the simulations, several hydrogen bonding motifs are found to persist at the onset of the simulations and over the time evolution of the structure dynamics that serve to effectively anchor the B_{12} at this loop region. Those which specifically anchor the B_{12} to this region in all simulations are shown in Figure 5, visualizing the two most persistent motifs for these structures: a pair of hydrogen bonds from a single B_{12} amide side chain to Pro8 and Glu16 (left, Motif 1) and an amide side chain to Pro8 hydrogen bond and coordination of a hydroxy group H atom on Ser13 to the B_{12} cyano nitrogen atom (right, Motif 2). The structural basis for preservation of the loop region itself across all simulations is evident in Figure 6, which shows that (left) Glu16 is engaged in several persistent hydrogen bonding interactions with Gly9, Glu10, and Asp11, whereas at the far end of the loop (right), Glu6 is in close proximity to hydrogen bond acceptors on Tyr27 and Ser23. The NMR distance lists, ranges, and time-averaged MD structures for **3** and **4** are provided in the Supporting Information.

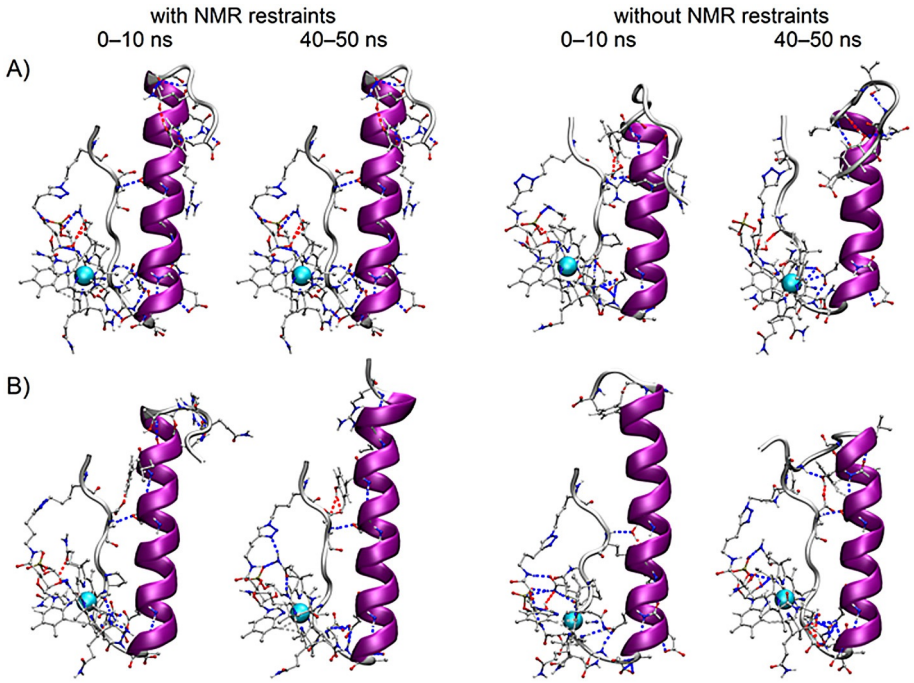


Figure 3. Side-on views (aligned along the α helix) of RMSD average structures (across 10 ns sampling increments) for restrained (left) and unrestrained (right) MD simulations of A) **3** and B) **4**.

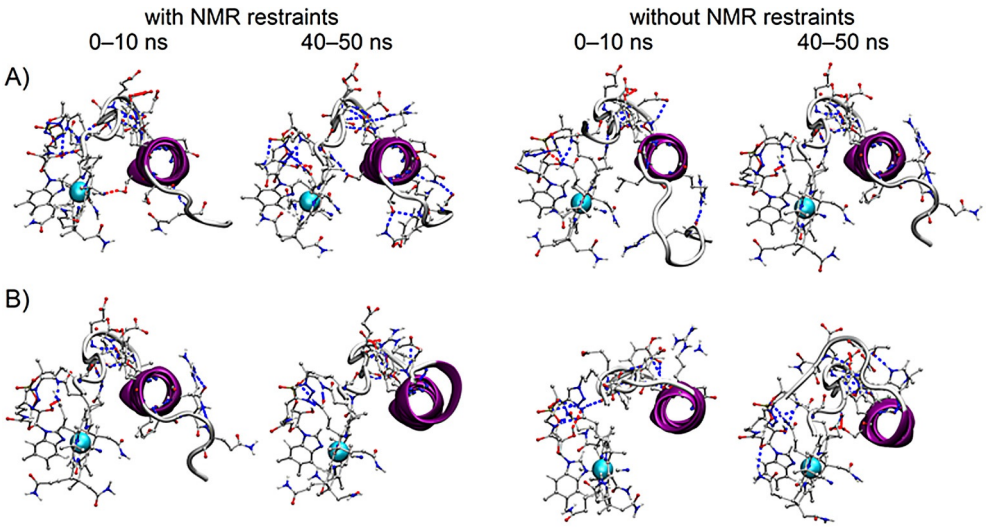


Figure 4. Top-down views (aligned along the α helix) of RMSD average structures (across 10 ns sampling increments) for restrained (left) and unrestrained (right) MD simulations of A) **3** and B) **4**.

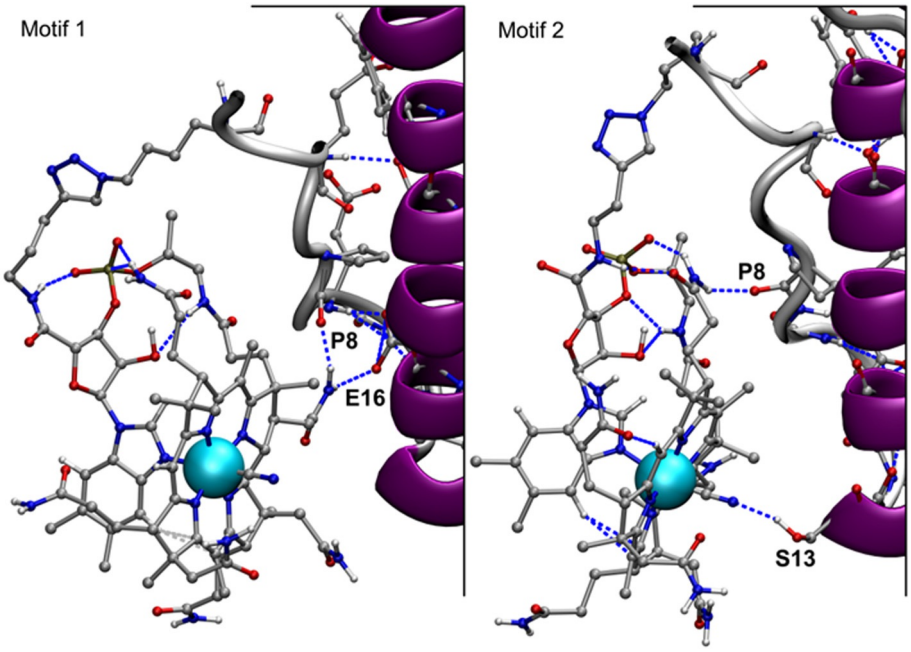


Figure 5. Two persistent hydrogen bonding interaction motifs between the B_{12} and PYY(3–36) unstructured region (residues 3–10) across all simulations.

The differences between structures **3** and **4**, both with and without NMR constraints, are largely localized to the C-terminal side of the α helix and occur to varying degrees in all of the simulations. With the B_{12} largely predicted to be confined to the PYY(3–36) loop due to several strong hydrogen bonding interactions, and with the well-known attenuation of PYY(3–36) activity with modifications to or removal of the C-terminal region, the most logical explanation for any change in behavior to come from these single conjugate simulations is some structural change at the C-terminal region of PYY(3–36). This may occur from B_{12} binding-induced conformational changes at the PYY N terminus, causing changes in activity, meaning any observed interactions between the B_{12} binding-constrained N terminus and concomitantly proximal C-terminal regions are of great interest. Despite the small change in tether length for these two cases, RMSD analyses and average structure generation produced two distinct structures that, for each case, revealed binding interactions deemed consistent with the trends in activity.

MD simulations of **3** and **4** highlight hydrogen bonding interactions that may govern the differences observed in Y2-R agonism and subsequent calcium mobilization and inhibitory cAMP effects. PYY(3–36) does not tolerate any interaction at the C terminus with respect to Y2-R stimulation.[32] If the

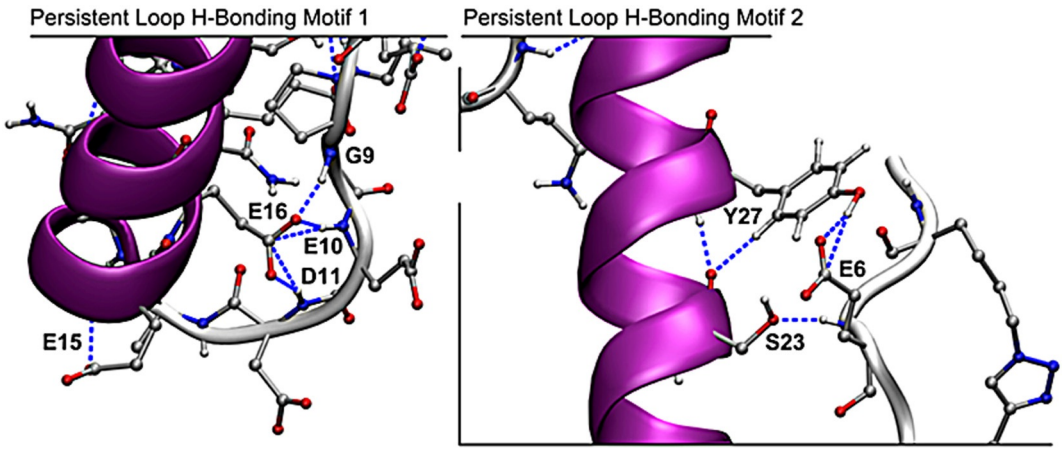


Figure 6. Two persistent hydrogen bonding motifs that define the PYY(3–36) loop region (left) and stabilizing interactions between the unstructured N-terminal region and the α helix.

answer to the decreased activity of **3** lies solely on some response internal to the conjugate, one might argue from the MD simulations that the shortened tether length enhances the stiffness of the unstructured region (residues 3–9) by decreasing its conformational flexibility upon hydrogen bonding between the B_{12} and the near-loop region. This result would provide a less flexible N-terminal region and a more persistent hydrogen bonding pocket for the C-terminal region with which to interact. By the loss of flexibility, necessary at the C-terminal region for biological activity, reduced activity would be predicted (and is observed). Kaiser et al. recently reported data showing that unwinding of C-terminal residues of neuropeptide Y (NPY) is critical for Y2 receptor binding and activation.[32] Solution NMR experiments showed that the ligand is tethered to the second extracellular loop by hydrophobic contacts and revealed NPY to undergo remarkable structural changes within the C terminus. The C-terminal pentapeptide plays a role in extensive and susceptible interactions in NPY; a network that is also relevant for PYY(3–36) in regards to Y2-R agonism. Changes in the C-terminal amino acids can easily disturb receptor binding or switch receptor selectivity for both NPY and PYY(3–36) as observed in numerous earlier structure-activity studies.[33] The ultimate conclusion from Kaiser et al. directly relates to our work, as the binding mode of NPY [and in our case, B_{12} conjugates of PYY(3–36)] might have more general implications for peptide binding GPCR systems.

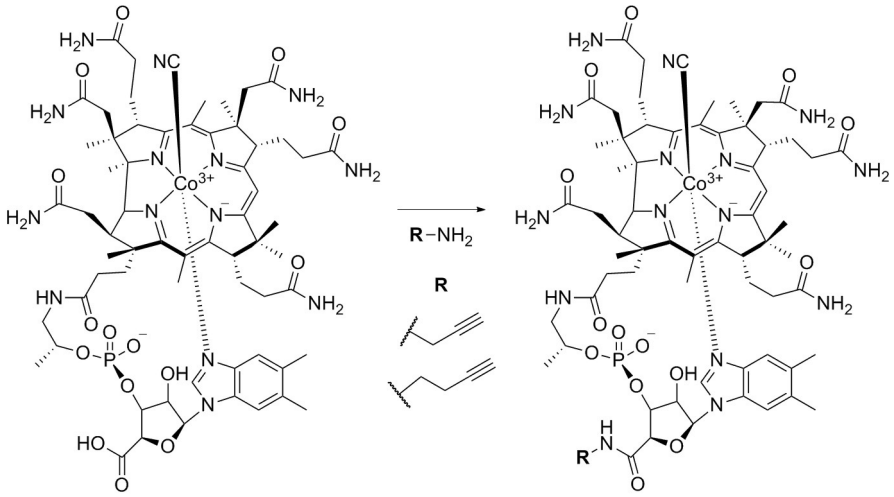
The MD simulations from this study do indicate that if the origin of the decreased activity of **3** is entirely due to factors internal to the B_{12}–PYY(3–36) conjugate itself, then constraint of the C-terminal region by hydrogen bonding interactions with the N-terminal region could explain it—and that this kind of internal mechanism may have its origin in the reduction of tether length.

Conclusions

B_{12}–PYY(3–36) conjugates **3** and **4** with various methylene spacer lengths between the B_{12} and PYY(3–36) show similar Y2-R agonism to that of PYY(3–36). Both intra- and intermolecular interactions between B_{12} and the peptide and small changes in the secondary structure of the peptide brought on by conjugation were observed. Based on the information collected from the NMR constrained MD studies, it would have been possible to offer a detailed assessment of the potential function of both conjugates. These observations suggest that MD could be used a priori to guide conjugate rational design and minimize the number of conjugates that would need to be screened—information of considerable benefit in development terms. Conjugates and modifications of B_{12} have garnered much interest in recent years for their clinical and medicinal applicability.[34] Based on the studies described herein, an ideal B_{12}–peptide conjugate would be one with an appropriate linker length to allow optimal function of both the peptide and B_{12}, which could be predicted by MD via inter- and intramolecular interactions that are known to be useful and/or harmful to the overall function of each component.

Experimental Section

Synthesis of alkyne precursors (1 and 2) and conjugates (3 and 4): Two B_{12}–alkyne precursors were prepared by activation of a B_{12}–carboxylic acid (B_{12}-CA) derivative[35] with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt) in anhydrous DMSO under argon (Scheme 1). Full characterization of the alkyne precursor **1** and **2**, including RP-HPLC, MALDI-ToF MS, and NMR can be found in the Supporting Information (Figure S1–11, Table S1). For conjugate synthesis, click chemistry[36] was implemented using a copper iodide (CuI) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) method, adapted from Gryko et al.[37,38] Copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC) synthesis of conjugates **3** and **4** via the alkyne precursors **1** and **2** and a K_4-azido PYY(3–36) (K_4PYY) is described in Scheme 2 (spacer length $n = 2, 3$ for precursors 1 and 2). K_4PYY was initially tested against PYY(3–36) amide (Sigma–Aldrich), and there was no observed difference in Y2-R agonism. Subsequently, K_4PYY was used as the control for all assays. Characterization of the B_{12}-PYY(3–36) conjugates **3** and **4**, including HPLC and MALDI-ToF MS, can be found in the Supporting Information (Figure S13–16).



Scheme 1. Synthesis of B_{12}–alkyne precursors **1** and **2**. *Reagents and conditions*: EDC, HOBt, anhydrous DMSO; reactions carried out under argon for 16 h at RT.

In vitro assay of 3 and 4 at the Y2 receptor coupled to G_i: Conjugates **3** and **4** were tested for their abilities to lower levels of cAMP in an in vitro assay using HEK293 cell monolayers (Figure 7).[29] These cells were engineered so that they stably express the human GLP-1 receptor (GLP-1R), while also transiently expressing the human Y2-R. Furthermore, these cells were virally transduced with the genetically encoded FRET reporter AKAR3, which is used to monitor cAMP-dependent protein kinase (PKA) activation intracellularly. This assay is unique in that it allows FRET-based detection of the ability of PYY(3–36) to counteract the action of a GLP-1R agonist (Exendin-4; Ex-4) to raise levels of cAMP. When the cells are first treated with Ex-4 (33 pM; injection 1) so that levels of cAMP are elevated, AKAR3 exhibits increased FRET, measured as a decrease in the 485/535 nm emission ratio (Figure 7). This change of FRET occurs after an approximate lag time of 50 s. If PYY(3–36) is then applied at the 180 s time point (injection 2), a functional antagonism of the action of Ex-4 is measured so that the change of FRET is decreased. Note that no change of FRET is measured in response to the administration of a negative control standard extracellular saline (SES; Figure 7). By varying the concentration of added conjugate, it is possible to determine dose–response relationships, and to also determine EC_{50} values describing inhibitory actions of PYY(3–36) conjugates versus 33 pM Ex-4 in this assay. Figure 7 illustrates these responses to the first injection of either Ex-4 or SES, and the second injection of K_4PYY or SES. To normalize these raw data for subsequent dose–response analysis, "end-point" values of FRET were measured during the last 10 sample intervals (Figure 7). As illustrated in Figure 1, the dose-dependent inhibitory actions of PYY(3–36) conjugates are then quantified relative to a value of 100% that corresponds to the maximal inhibitory effect measured when testing 300 nM PYY(3–36) in this assay.

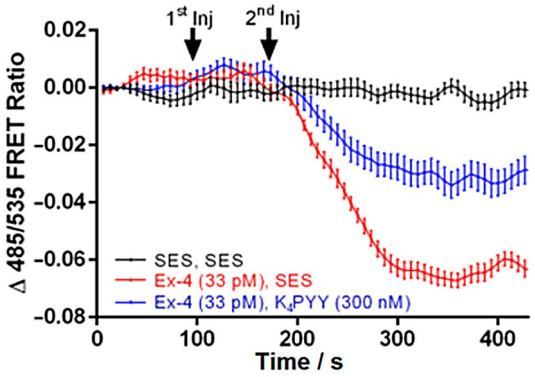
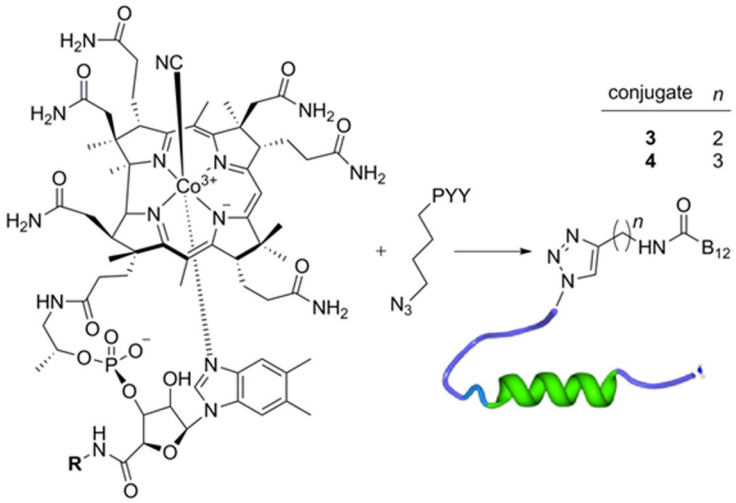


Figure 7. Δ485/535 FRET ratio through the course of a run. The first injection occurs at 100 s followed by a second injection at 180 s.



Scheme 2. Copper(I) alkyne–azide cycloaddition synthesis of B_{12}–PYY(3–36) conjugates **3** and **4** via alkyne precursors **1** and **2** with a two- or three-methylene-unit spacer between the 5′-amide on B_{12} and the triazole linkage to K_4PYY. *Reagents and conditions*: Cu^{I}, TBTA; reactions were carried out for 16 h at RT. PYY(3–36) adapted from PDB ID: 2DF0.

NMR studies of 3 and 4: NMR studies were executed initially to observe any structural differences between the conjugates and free peptide in solution. Because in vivo studies had previously established in vivo function for **3**, extensive structural studies were first performed with **3** and a direct comparison made to published PYY(3–36).[7] Full NMR studies of **4** were then completed to serve as

a comparison with **3**. Full descriptions of methods and conditions are provided in the Supporting Information.

MD studies of 3 and 4: To complement the NMR studies, MD simulations of **3** and **4** were completed in attempts to explain the minor differences of Y2-R agonism between the two conjugates. These MD studies took the form of 50 ns simulations to probe the potential variation in simulation geometries and time-averaged structures that arise from different tether lengths. MD simulations were performed using the GROMACS (ver. 5.0.4)[39] software package. The NMR distance lists, ranges, and tabulated distances from the time-averaged MD structure for **3** are provided in the Supporting Information.

Acknowledgements

This research was supported by the National Institute of Diabetes and Digestive and Kidney Diseases of the US National Institutes of Health (NIH) under award number R15K097675-01A1. The content is solely the responsibility of the authors and does not necessarily represent the official views of the US NIH. The authors thank the Syracuse University HTC Campus Grid, supported by US National Science Foundation (NSF) award ACI-1341006, for computational resource use.

Keywords: GPCR · molecular dynamics · NMR spectroscopy · PYY(3–36) · vitamin B_{12}

[1] C. Martins, L. M. Morgan, S. R. Bloom, M. D. Robertson, *J. Endocrinol.* **2007**, *193*, 251 – 258.
[2] E. Ekblad, F. Sundler, *Peptides* **2002**, *23*, 251 – 261.
[3] E. Karra, R. L. Batterham, *Mol. Cell. Endocrinol.* **2010**, *316*, 120 – 128.
[4] D. Larhammar, *Regul. Pept.* **1996**, *62*, 1 – 11.
[5] C. Cabrele, A. G. Beck-Sickinger, *J. Pept. Sci.* **2000**, *6*, 97 – 122.
[6] A. G. Blomqvist, C. Söderberg, I. Lundell, R. J. Milner, D. Larhammar, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 2350 – 2354.
[7] R. Nygaard, S. Nielbo, T. W. Schwartz, F. W. Poulsen, *Biochemistry* **2006**, *45*, 8350 – 8357.
[8] B. Bjoernholm, F. S. Joergensen, T. W. Schwartz, *Biochemistry* **1993**, *32*, 2954 – 2959.
[9] K. Tatemoto, V. Mutt, *Nature* **1980**, *285*, 417 – 418.
[10] T. H. Moran, U. Smedh, K. P. Kinzig, K. A. Scott, S. Knipp, E. E. Ladenheim, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2005**, *288*, R384 – R388.
[11] L. Degen, S. Oesch, M. Casanova, S. Graf, S. Ketterer, J. Drewe, C. Beglinger, *Gastroenterology* **2005**, *129*, 1430 – 1436.
[12] R. L. Batterham, H. Heffron, S. Kapoor, J. E. Chivers, K. Chandarana, H. Herzog, C. W. Le Roux, E. L. Thomas, J. D. Bell, D. J. Withers, *Cell Metab.* **2006**, *4*, 223 – 233.
[13] D. Grandt, M. Schimiczeka, Ch. Beglinger, P. Layera, H. Goebella, V. E. Eysselein, J. R. Reeve, Jr., *Regul. Pept.* **1994**, *51*, 151 – 159.
[14] R. Mentlein, P. Dahms, D. Grandt, R. Krüger, *Regul. Pept.* **1993**, *49*, 133 – 144.
[15] L. A. Selbie, K. Darby, C. Schmitz-Peiffer, C. L. Browne, H. Herzog, J. Shine, T. J. Biden, *J. Biol. Chem.* **1995**, *270*, 11789 – 11796.
[16] P. Sjödin, S. K. Holmberg, H. Åkerberg, M. M. Berglund, N. Mohell, D. Larhammar, *Biochem. J.* **2006**, *393*, 161 – 169.
[17] C. R. Abbott, M. Monteiro, C. J. Small, A. Sajedi, K. L. Smith, J. R. Parkinson, M. A. Ghatei, S. R. Bloom, *Brain Res.* **2005**, *1044*, 127 – 131.
[18] S. Koda, Y. Date, N. Murakami, T. Shimbara, T. Hanada, K. Toshinai, A. Niijima, M. Furuya, N. Inomata, K. Osuye, M. Nakazato, *Endocrinology* **2005**, *146*, 2369 – 2375.
[19] J. E. Blevins, P. K. Chelikani, A. C. Haver, R. D. Reidelberger, *Peptides* **2008**, *29*, 112 – 119.
[20] N. M. Neary, C. J. Small, M. R. Druce, A. J. Park, S. M. Ellis, N. M. Semjonous, C. L. Dakin, K. Filipsson, F. Wang, A. S. Kent, G. S. Frost, M. A. Ghatei, S. R. Bloom, *Endocrinology* **2005**, *146*, 5120 – 5127.
[21] K. E. Henry, C. T. Elfers, R. M. Burke, O. G. Chepurny, G. G. Holz, J. E. Blevins, C. L. Roth, R. P. Doyle, *Endocrinology* **2015**, *156*, 1739 – 1749.
[22] J. F. McEwan, H. S. Veitch, G. J. Russell-Jones, *Bioconjugate Chem.* **1999**, *10*, 1131 – 1136.
[23] A. K. Petrus, A. R. Vortherms, T. J. Fairchild, R. P. Doyle, *ChemMedChem* **2007**, *2*, 1717 – 1721.
[24] C. H. Fazen, D. Valentin, T. J. Fairchild, R. P. Doyle, *J. Med. Chem.* **2011**, *54*, 8707 – 8711.
[25] S. Clardy-James, O. G. Chepurny, C. A. Leech, G. G. Holz, R. P. Doyle, *ChemMedChem* **2013**, *8*, 582 – 586.
[26] S. L. Pedersen, B. Holst, N. Vrang, K. J. Jensen, *J. Pept. Sci.* **2009**, *15*, 753 – 759.
[27] S. L. Pedersen, P. G. Sasikumar, S. Chelur, B. Holst, A. Artmann, K. J. Jensen, N. Vrang, *J. Pept. Sci.* **2010**, *16*, 664 – 673.
[28] S. L. Pedersen, C. Steentoft, N. Vrang, K. J. Jensen, *ChemBioChem* **2010**, *11*, 366 – 374.
[29] G. G. Holz, C. A. Leech, M. W. Roe, O. G. Chepurny in *Cyclic Nucleotide Signaling* (Ed.: Xiaodong Cheng), Taylor and Francis Group, CRC Press, Boca Raton, **2015**, 35 – 60.
[30] D. A. Keire, M. Kobayashi, T. E. Solomon, J. R. Reeve, *Biochemistry* **2000**, *39*, 9935 – 9942.
[31] E. F. G. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J. Comput. Chem.* **2004**, *25*, 1605 – 1612.
[32] A. Kaiser, P. Müller, T. Zellmann, H. A. Scheidt, L. Thomas, M. Bosse, R. Meier, J. Meiler, D. Huster, A. G. Beck-Sickinger, P. Schmidt, *Angew. Chem. Int. Ed.* **2015**, *54*, 7446 – 7449; *Angew. Chem.* **2015**, *127*, 7554 – 7558.
[33] X. Pedragosa Badia, J. Stichel, A. G. Beck-Sickinger, *Front. Endocrinol.* **2013**, *4*, 5.
[34] F. Zelder, *Chem. Commun.* **2015**, *51*, 14004 – 14017.
[35] S. Clardy-James, J. Bernstein, D. Kerwood, R. P. Doyle, *Synlett* **2012**, *23*, 2363 – 2366.
[36] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004 – 2021; *Angew. Chem.* **2001**, *113*, 2056 – 2075.
[37] M. Chromiński, D. Gryko, *Chemistry* **2013**, *19*, 5141 – 5148.
[38] K. ó Proinsias, M. Giedyk, D. Gryko, *Chem. Soc. Rev.* **2013**, *42*, 6605 – 6619.
[39] B. Hess, C. Kutzner, D. van der Spoel, E. Lindahl, *J. Chem. Theory Comput.* **2008**, *4*, 435 – 447.

Received: February 2, 2016
Published online on March 30, 2016

TITLE

COAGONISTS OF GLUCAGON-LIKE PEPTIDE 1 RECEPTOR AND NEUROPEPTIDE Y2 RECEPTOR

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

[0001] The present invention relates to peptides for treating weight loss and glucose levels and, more specifically, to a single peptide that can trigger weight loss while controlling glucose levels.

2. DESCRIPTION OF THE RELATED ART

[0002] The glucagon-like peptide 1 receptor (GLP-1R) is involved in stimulating the release of insulin in a glucose dependent manner. As a result, GLP-1R agonists have been the source of development for drugs that can be used to treat insulin deficiency diseases such as diabetes. The neuropeptide Y2 receptor (NPYR2) is involved in appetite signaling and suppression. Accordingly, there is a need in the art for a drug that could serve as an agonist of both receptors, thereby more fully addressing the problems associated with glucose regulation and food intake.

[0003] BRIEF SUMMARY OF THE INVENTION

[0004] The present invention comprises peptide sequences that can serve as agonists of both the glucagon-like peptide 1 receptor (GLP-1R) and the neuropeptide Y2 receptor (NPYR2). In particular, the invention comprises certain non-naturally occurring peptides that can successfully serve as agonists for both receptors. The present invention may also be used as a coagonist at other receptors, such as the NPYR1 or NPYR4 or NPYR5, and not necessarily exclusively GLP-1R and NPYR2.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0005] The present invention will be more fully understood and appreciated by reading the following Detailed Description in conjunction with the accompanying drawings, in which:

[0006] FIGS. 1A and 1B are graphs of the results of agonism screening for certain peptides according to the present invention;

[0007] FIGS. 2A and 2B are graphs of the results of agonism screening for certain peptides according to the present invention;

[0008] Fig. 3 is a chart of GLP-1R agonism screening for certain peptides at a concentration of 100nM;

[0009] Fig. 4 is a chart of NPYR2 agonism screening for certain peptides at a concentration of 100nM;

[0010] Fig. 5 is a graph of GLP-1R agonism of an exemplary peptide according to the present invention;

[0011] Fig. 6 is a graph of NPYR2 agonism of an exemplary peptide according to the present invention;

[0012] Fig. 7 shows the comparison of Ex-4 and PYY and highlights the critical components of each; and

[0013] Fig. 8 shows potential variations of RLB001 that may have comparable or better function at both the GLP-1R and NPYR2.

DETAILED DESCRIPTION OF THE INVENTION

[0014] Referring now to the drawings, wherein like reference numerals refer to like parts throughout, the present inventions comprises peptide sequences that are coagonists of GLP-1R and NPYR2. The sequences of the present invention were designed by combining certain

aspects of the natural substrates or known agonists of GLP-1R and NPYR2., such as Peptide YY and exendin-4.

[0015] Following the approach of the present invention, the sequences HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQRY-NH2 (SEQ. ID NO. 1), referred to herein as Peptide RLB001, HGEGTFTSDLSKQMEEEAVRLFIEWLRHYLNLVTRQRY-NH2 (SEQ. ID NO. 2), referred to herein as Peptide RLB002, and IKPEAPREDASPEEENQAYKEFIAYLNLVTRQRY-NH2 (SEQ. ID. NO. 3), referred to herein as Peptide RLB003 were designed and synthesized through solid phase peptide synthesis.

[0016] Referring to FIGS. 1A and 1B, the sequences were screened for agonism with respect to each of GLP-1R and NPYR2 by performing a either a Fluorescence Resonance Energy Transfer (FRET) assay to show GLP-1R agonism or following the fluorescence of FURA 2 upon calcium release to show NPYR2 agonism and plotting the change in either FRET or fluorescence over a predetermined time period. These assays have been used previously to show function at each receptor respectively. The peptides synthesized according to the present invention were compared against a basal measurement, exendin-4 (a known agonist of GLP-1R), Peptide YY (a known agonist of NPYR2), and ionomycin.

[0017] Referring to FIGS. 2A and 2B, Peptide RLB001 was screened for agonism with respect to each of GLP-1R and NPYR2, as well as the controls discussed above. Referring to FIG. 3, screening of the peptides for GLP-1R agonism revealed that RLB001 and RLB002 were effective agonists. Referring to FIG. 4, screening of the peptides for NPYR2 agonism revealed that RLB001 was even more effective that known agonists of NPY2R, such as PYY. As seen in FIG. 5, Peptide RLB001 done in triplicate has an EC_{50} of 50 pM at GLP-1R. As seen in FIG. 6, Peptide RLB001 done in duplicate has an EC_{50} of 81 nM at NPYR2. The relevant portions of

PYY and exendin-4 used as a basis for forming the sequences according to the present invention are seen in FIG. 7. Referring to FIG. 8, SEQ ID NO. 4 through SEQ ID NO. 12 represent variations of RLB001 that may have comparable or better function at both the GLP-1R and NPYR2 and may be readily tested as described above to confirm their efficacy. The present invention may also be used as a coagonist at other receptors, such as the NPYR1 or NPYR4 or NPYR5, and not necessarily exclusively GLP-1R and NPYR2

[0018] These results demonstrate that the peptide RLB001 has comparable function at the GLP-1R and the NPYR2. A single peptide may thus be used to activate two receptors responsible for glucose control and appetite suppression. In addition, the unique profile of calcium release after activation at the NPYR2 may indicate biased agonism.

[0019] Sequences according to the present invention may be further modified according to known processes, such as glycosylation, to improve the use of the sequences as pharmaceuticals by assisting with the delivery of the protein to a subject. For example, N-linked glycosylation may be used to attach oligosaccharides to a nitrogen atom, such as the N4 of asparagine residues. Similarly, O-linked glycosylation may be used to attach glycans to serine and threonine and C-linked glycosylation used for the covalent attachment of a mannose residue to a tryptophan residue.

[0020] Sequences according to the present invention may be further modified by pegylation, which is the attachment of a therapeutic protein to poly(ethylene glycol) polymer chains (PEG). The attachment of poly(ethylene glycol) chains can prevent degradation by proteolytic enzymes, reduce rapid clearance of the sequences by the kidneys, and increase the circulating half-life. As is known in the art, PEG is linked to a protein sequence through reactive molecular groups on amino acid side chains such as lysine.

[0021] Sequences according to the present invention may be further modified by lipidation. The presence of a lipid group in peptides modulates their hydrophobicity, secondary structures and self-assembling propensities while retaining their abilities to bind to target receptors. Lipidation improves metabolic stability, membrane permeability, bioavailability, and changes pharmacokinetic and pharmacodynamic properties of peptides.

[0022] The sequences may also be encapsulated in a suitable vehicle to either aid in the delivery of the compound to target cells, to increase the stability of the composition, or to minimize potential toxicity of the composition. For example, nanoparticles, liposomes, microemulsions, micelles, dendrimers and other phospholipid-containing systems may be used as is known in the art.

[0023] Sequences according to the present invention may further be conjugated to known conjugation partners to assist in the use of the sequences as a pharmaceutical. For example, conjugation partners such as an organic drug molecule, an enzyme label, a toxin, a cytostatic agent, a label which can be photoactivated and which is suitable in photodynamic therapy, a pharmaceutically suitable radioactive label, a hapten, digoxigenin, biotin, a chemotherapeutic metal complex or metal, colloidal gold, or a moiety that extends the serum half-life may be used to assist with the delivery of the present invention.

[0024] The present invention includes pharmaceutical compositions comprising a preparation of the sequences of the invention. Such pharmaceutical compositions may be for administration for injection, or for oral, nasal, transdermal or other forms of administration, including, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, intraocular, retrobulbar, intrapulmonary (e.g., aerosolized drugs) or subcutaneous injection (including depot administration for long term release); by sublingual, anal, vaginal, or

by surgical implantation. The treatment may consist of a single dose or a plurality of doses over a period of time. In general, pharmaceutical compositions comprising effective amounts of a sequence according to the invention, whether modified as described above or not, together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content, pH and ionic strength; additives such as detergents and solubilizing agents, anti-oxidants, preservatives and bulking substances. The pharmaceutical compositions optionally may include still other pharmaceutically acceptable liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.

WHAT IS CLAIMED IS:

1. A composition for simultaneously treating obesity and insulin deficiency, comprising a peptide sequence having a first portion that corresponds to a selected portion of the sequence of an agonist of the glucagon-like peptide 1 receptor (GLP-1R) and a second potion that corresponds to a selected portion of the sequence of an agonist of the neuropeptide Y2 receptor (NPYR2).

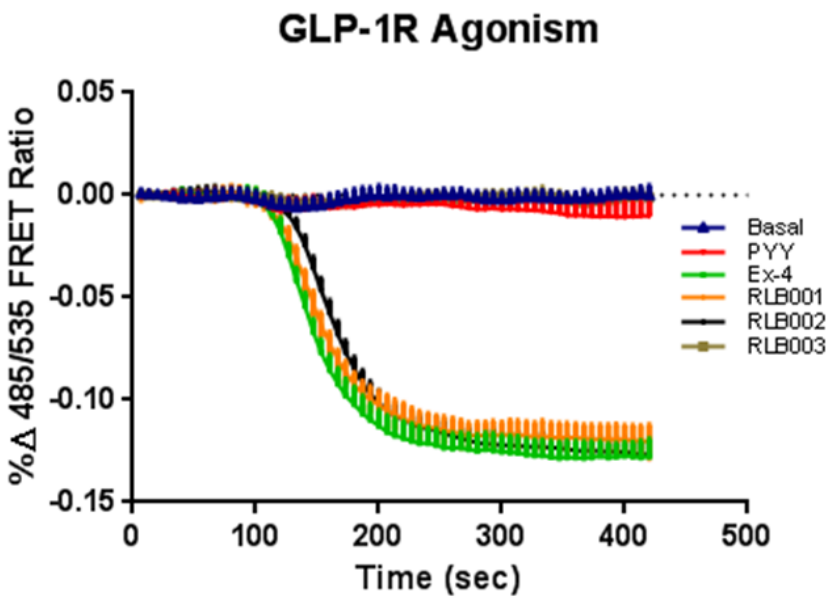
2. The composition of claim 1, wherein the sequence comprises HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQRY-NH2 (SEQ. ID NO. 1).

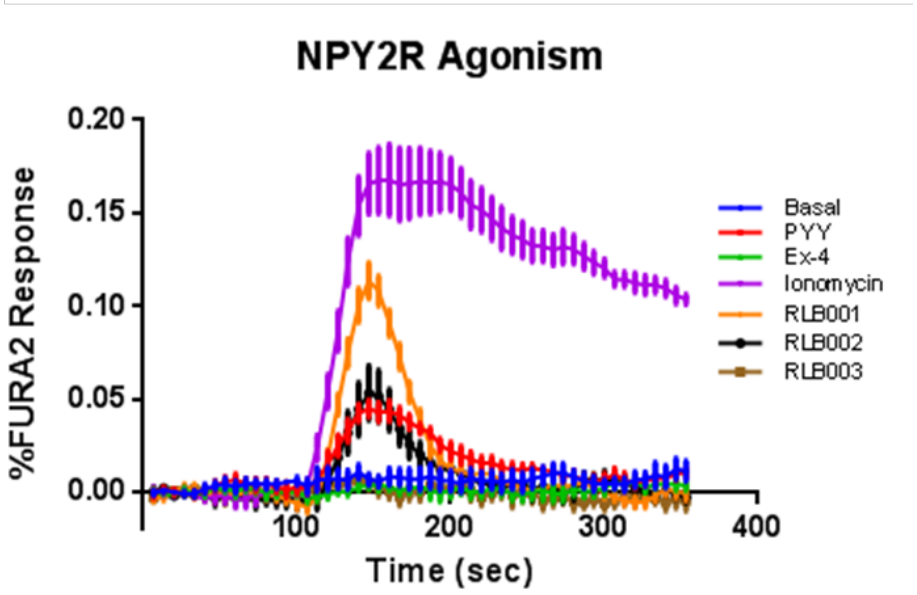
3. A method of simultaneously treating obesity and insulin deficiency, comprising the steps of administering a peptide sequence having a first portion that corresponds to a selected portion of the sequence of an agonist of the glucagon-like peptide 1 receptor (GLP-1R) and a second potion that corresponds to a selected portion of the sequence of an agonist of the neuropeptide Y2 receptor (NPYR2).

4. The method of claim 1, wherein the sequence comprises HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQRY-NH2 (SEQ. ID NO. 1).

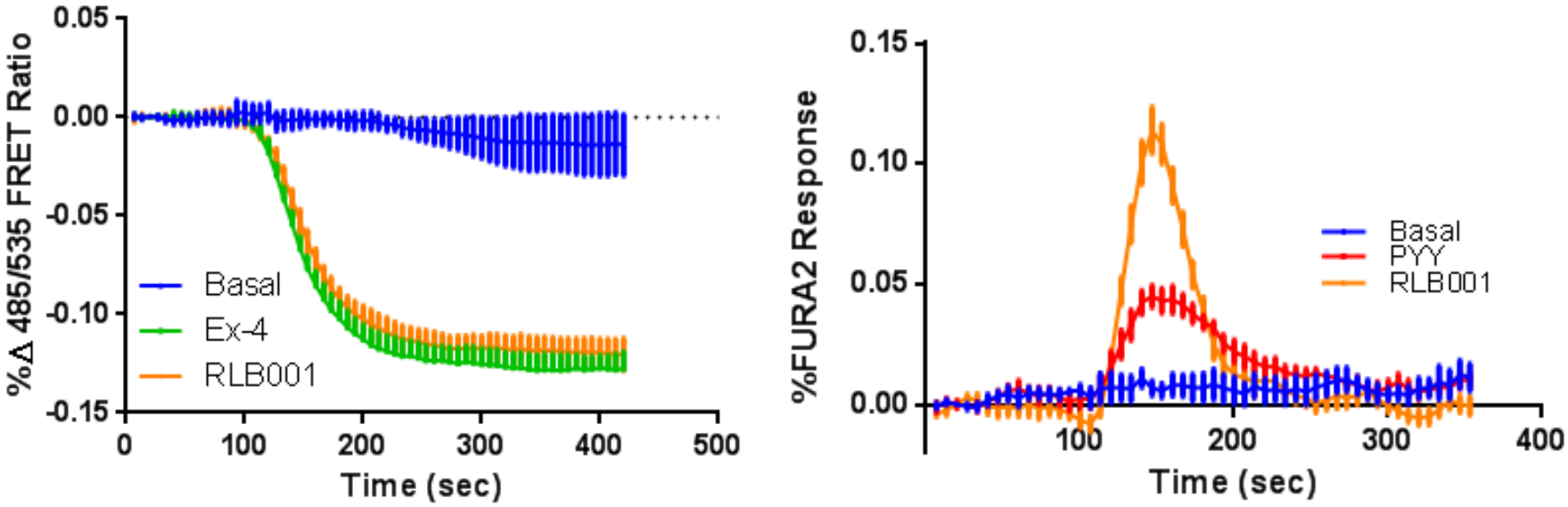
ABSTRACT

Peptide sequences that can serve as agonists of both the glucagon-like peptide 1 receptor (GLP-1R) and the neuropeptide Y2 receptor (NPYR2). The peptide sequences include regions that correspond to certain aspect of natural substrates and known agonists of the glucagon-like peptide 1 receptor (GLP-1R) and the neuropeptide Y2 receptor (NPYR2) in a single sequence.


GLP-1R Agonism
%Δ 485/535 FRET Ratio
0.05
0.00
-0.05
-0.10
-0.15
0
100
200
300
400
500
Time (sec)
Basal
PYY
Ex-4
RLB001
RLB002
RLB003


NPY2R Agonism
%FURA2 Response
0.20
0.15
0.10
0.05
0.00
100
200
300
400
Time (sec)
Basal
PYY
Ex-4
Ionomycin
RLB001
RLB002
RLB003

FIGS. 1A and 1B



FIGS. 2A and 2B

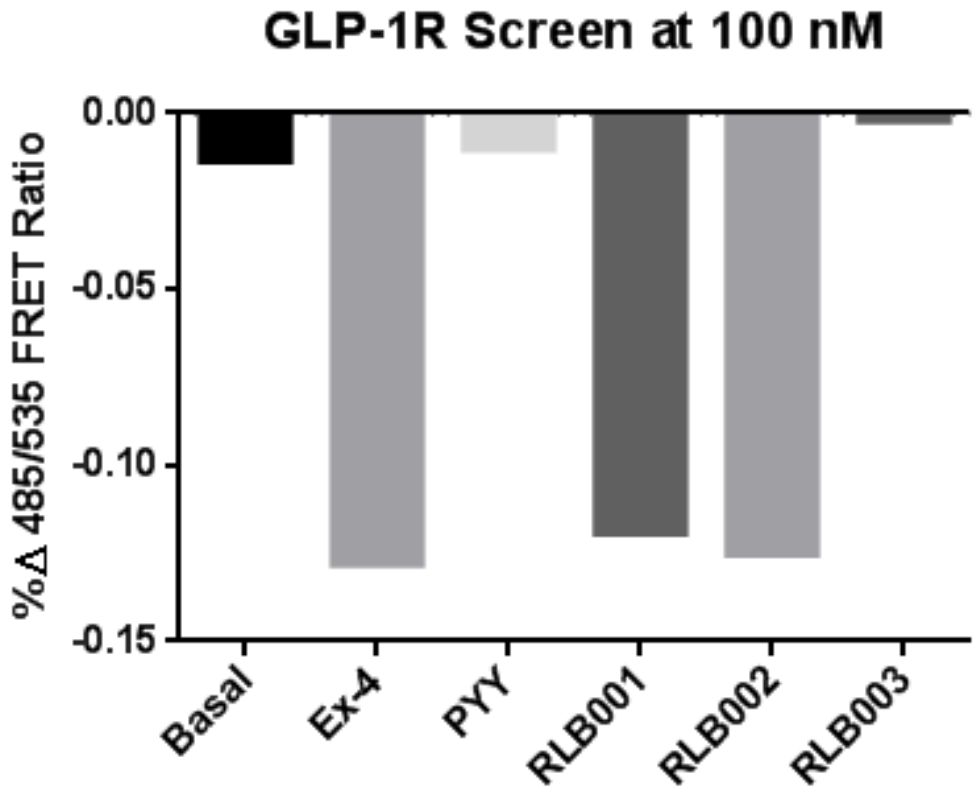

GLP-1R Screen at 100 nM
%Δ 485/535 FRET Ratio
0.00
-0.05
-0.10
-0.15
Basal
Ex-4
PYY
RLB001
RLB002
RLB003

FIG. 3

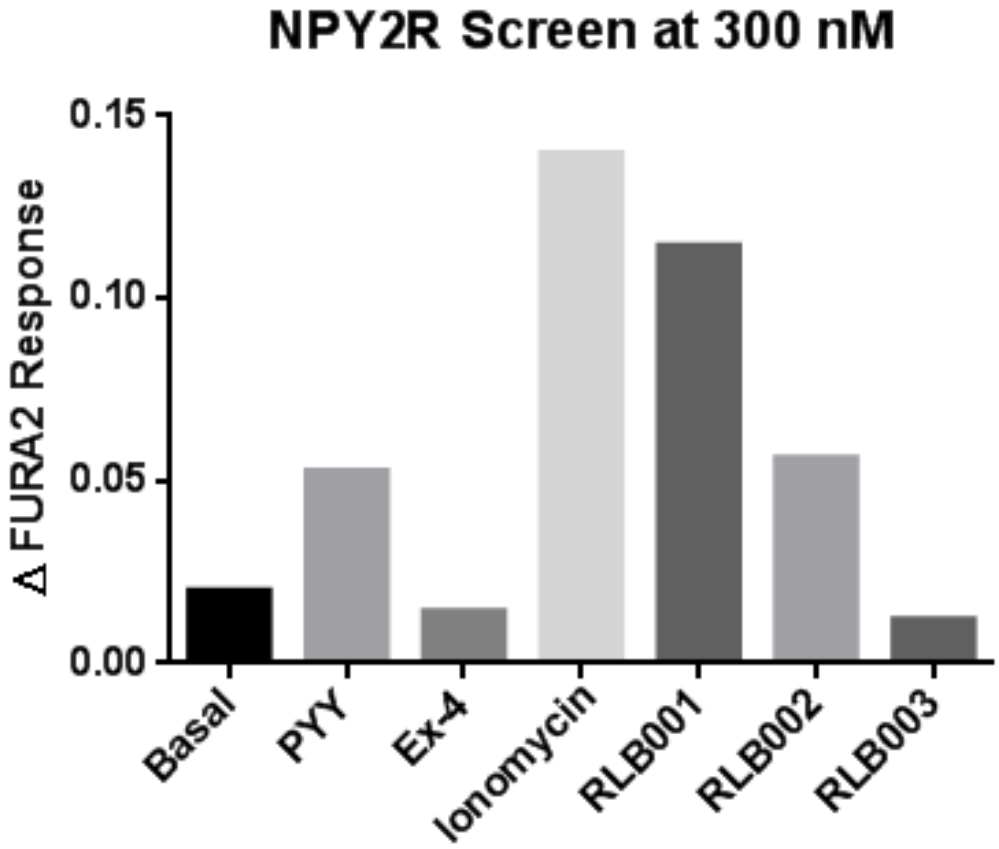

NPY2R Screen at 300 nM
Δ FURA2 Response
0.15
0.10
0.05
0.00
Basal
PYY
Ex-4
Ionomycin
RLB001
RLB002
RLB003

FIG. 4

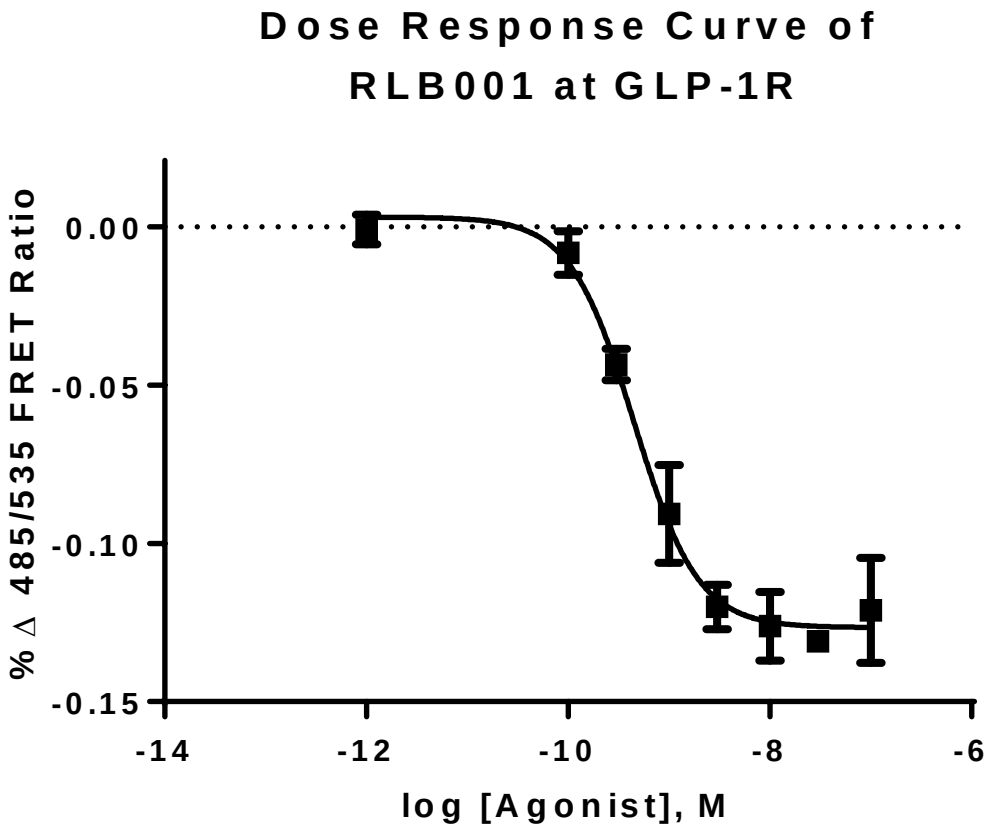

Dose Response Curve of
RLB001 at GLP-1R
% Δ 485/535 FRET Ratio
0.00
-0.05
-0.10
-0.15
-14
-12
-10
-8
-6
log [Agonist], M

FIG. 5

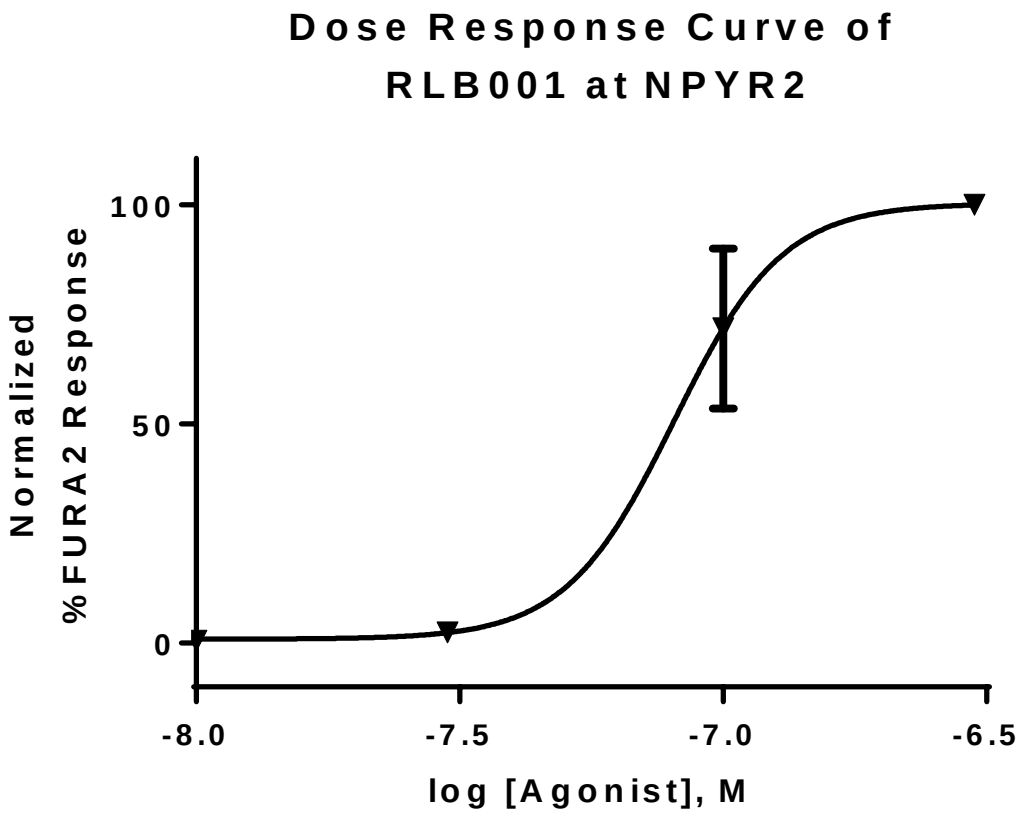

Dose Response Curve of
RLB001 at NPYR2
Normalized
%FURA2 Response
100
50
0
-8.0
-7.5
-7.0
-6.5
log [Agonist], M

FIG. 6

Ex-4	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH2
PYY	IKPEAPREDASPEELNRYYASLRHYLNLVTRQRY-NH2
RLB001	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSTRQRY-NH2
RLB002	HGEGTFTSDLSKQMEEEAVRLFIEWLRHYLNLVTRQRY-NH2
RLB003	IKPEAPREDASPEEENQAYKEFIAYLNLVTRQRY-NH2

FIG. 7

1	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGTRQRY-NH2 (SEQ. ID. NO. 4)
2	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGTRQRY-NH2 (SEQ. ID. NO. 5)
3	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGLRHYLNLVTRQRY-NH2 (SEQ. ID. NO. 6)
4	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRGTRQRY-NH2 (SEQ. ID. NO. 7)
5	HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRGTRQRY-NH2 (SEQ. ID. NO. 8)
6	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRGLRHYLNLVTRQRY-NH2 (SEQ. ID. NO. 9)
7	HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRGLRHYLNLVTRQRY-NH2 (SEQ. ID. NO. 10)
8	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSTRQRY-NH2 (SEQ. ID. NO. 11)
9	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPSLRHYLNLVTRQRY-NH2 (SEQ. ID. NO. 12)

Fig. 8

Vita

Ron Bonaccorso was born in Gainsville, FL in August of 1990. He grew up in Rochester, NY and then completed undergraduate work at Rochester Institute of Technology in 2012. In 2016 he completed his PhD in Chemistry at Syracuse University.